UNIVERSIDADE TÉCNICA DE LISBOA

INSTITUTO SUPERIOR DE AGRONOMIA

Divergência ecológica e genética numa população da processionária do pinheiro com desvio na sua fenologia

Ecological and genetic divergence in a population of the pine processionary moth with shifted phenology

Tese apresentada para obtenção do grau de Doutor em Engenharia Florestal e dos Recursos Naturais

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Helena Maria Gonçalves Santos

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Resumo

Analisa-se um caso de diferenciação genética e ecológica resultante de um desvio na fenologia na processionária do pinheiro. Duas populações simpátricas encontram-se isoladas reprodutivamente por desfasamento na emergência dos adultos. O desenvolvimento larvar é parcialmente simultâneo, embora os estádios do ciclo de vida ocorram em estações do ano distintas.

Monitorizou-se no campo o período de voo em vários anos e locais. Utilizaram-se marcadores genéticos para obter atribuição genética e diferenciação das populações de acordo com a fenologia. Os resultados evidenciaram elevada distância genética entre populações. A estrutura genética mostrou-se estável no tempo. No geral os indivíduos foram correctamente atribuídos à sua população de referência.

Estudou-se a diferenciação ecológica nos estádios de ovo e larva. Demonstrou-se uma maior tolerância a temperaturas superiores a 36ºC em larvas da população com fenologia desfasada. Observou-se ainda divergência ecológica na fecundidade, tamanho e cobertura dos ovos e no parasitismo óofago.

Estudaram-se em laboratório os padrões fenológicos dos adultos nas duas populações e resultantes híbridos, observando a heritabilidade genética da fenologia. Os resultados mostraram elevada heritabilidade da fenologia, tendo os híbridos características intermédias. Microsatélites permitiram discriminar os híbridos.

Em conclusão, os resultados indicam a presença de um processo de especiação alocrónica incipiente com diferenciação ecológica em curso.

Palavras chave: Especiação; Alocronia; processionária do pinheiro; divergência ecológica; genética; fenologia; Evolução saltacional; hibridação; adaptação; expansão

Abstract

Ecological and genetic divergence in a population of the pine processionary moth with shifted phenology

A case of incipient allochronic speciation through phenological shift in the pine processionary moth is studied. Two sympatric populations are reproductively isolated by the timing of adult emergence. Larval development is partially simultaneous, although life stages occur in different seasons.

Adult flight was monitored in the field for several years and locations. Genetic markers were used to obtain genetic assignment and population differentiation according to the phenology. Results evidence high genetic distance between the two populations. The genetic structure revealed to be stable in time. In general, sampled individuals could be correctly assigned to their reference populations.

Ecological differentiation was assessed in the egg and larval stages. Larvae from the shifted population revealed higher tolerance to temperatures above 36ºC. Ecological divergence was observed in the egg stage, namely in load, size, coverage and parasitism .

The phenology patterns of adult emergence were studied on the two sympatric populations and their artificially obtained hybrids, aiming at determining phenotypic heritability of the emergence pattern. Results indicate high heritability, and the hybrids reveal intermediate adult phenology. Microsatellite genotyping allowed the discrimination of hybrids.

Results here presented clearly show the occurrence of an incipient allochronic speciation process with ecological differentiation in course.

Keywords: speciation; Allochrony; pine processionary moth; Ecological divergence; genetics; phenology; saltational evolution; hybridization; adaptation; expansion

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Aos meus amigos e colegas do departamento por quem tenho a maior consideração, à Xana, à Marta, ao António, obrigada por estarem sempre lá.

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1. INTRODUCTION

1. Introduction

This work regards the study of evolutionary and ecological consequences of a change in phenology in a population of the pine processionary moth *Thaumetopoea pityocampa*, which is experiencing a process of allochronic differentiation. With this introduction we aim to present a brief synthesis of the allochronic speciation processes, of the pine processionary moth and its variability, in particular regarding phenology, and of Leiria area and its specificity. Finally the objectives of the present work are presented.

Allochronic speciation

The diversification of the number of species in sexually reproducing animals is usually accepted to occur as described by Mayr (1942), where populations become geographically isolated and are led to diverge through differential selection and the accumulation of chance mutations and recombination. The process by which species diverge into different strains that become reproductively isolated and evolutionarily independent is well-known and documented for numerous cases of spatial isolation known as allopatric speciation (e.g Johannesson, 2001; Turelli *et al*., 2001). Nevertheless, speciation can occur in different scenarios than just the simple spatial separation of populations. Ecological shifts, such as the invasion of novel habitats, host shifts, or climate changes can result in rapid rates of divergence between populations (Orr & Smith, 1998).

The study of speciation is yet controversial. Ever since the 19th century the possibility of speciation to occur without geographical separation has been debated (Maynard Smith, 1966; Kondrashov & Mina, 1986; Bush, 1994; Bush & Smith, 1998; Dieckmann & Doebeli, 1999; Turelli *et al*., 2001; Via, 2001; Berlocher & Feder, 2002; Fry, 2003; Gavrilets, 2003; Feder *et al*., 2005; Gavrilets, 2006; Bolnick & Fitzpatrick, 2007; Butlin *et al*., 2008; Fitzpatrick *et al*., 2008). Speciation in sympatry had some supporters, but lacked convincing empirical evidence (Maynard Smith, 1966; Futuyma & Mayer, 1980; Fry, 2003; Fitzpatrick *et al*., 2008) and the proposed models require conditions that are difficult to obtain in natural populations (Bolnick & Fitzpatrick, 2007). Nevertheless, in the last 20 years several studies, both in animals and plants,

have come to show that sympatric speciation is in fact an acceptable premise and a few case studies have shown that it could be more common than originally thought (Bush & Smith, 1998). Sympatric speciation occurs whenever physical or temporal barriers lead to reproductive isolation while the different populations still occur in the same area. Host or habitat specialization is one of the most documented processes leading to sympatric isolation and speciation, mostly in fishes and phytophagous insects (e.g Feder & Filchak, 1999; Filchak *et al*., 2000; Cronin & Abrahamson, 2001; Berlocher & Feder, 2002; Drès & Mallet, 2002; Teixeira & Polavarapu, 2003; Favret & Voegtlin, 2004; Blair *et al*., 2005; Feder *et al*., 2005c), although non-random mating by sexual selection (Turner & Burrows, 1995) or migratory routes in birds (Bearhop *et al*., 2005) are also appointed as leading to sympatric speciation.

Sympatric speciation can also occur because of reproductive isolation in time, a process known as allochronic speciation (Alexander & Bigelow, 1960). Such situations can result from phenological incompatibilities that lead to different reproductive times, when populations reproduce in different years as observed in periodical Cicadas (Marshall & Cooley, 2000; Simon *et al*., 2000; Cooley *et al.*, 2001; Ritchie, 2001), or in different seasons of the year as for field crickets (Alexander & Bigelow, 1960) and a seabird species (Friesen *et al*., 2007), or even in different times of the day like in a Diptera species (Miyatake *et al*., 2002). In many insect groups, the adult stage is short-lived, thus small differences in phenology can prevent adults occurring in the same area from mating. Reproductive isolation can occur if there are differential non-overlapping reproductive seasons and if the reproductive phenology is heritable. Very few cases of allochronic speciation due to phenological isolation have been studied so far (Teixeira & Polavarapu, 2003; Abbot & Withgott, 2004; Santos *et al*., 2007) probably due to its scarcity. The pine processionary moth thus constitutes a documented study case, where a phenological shift caused an allochronic speciation process (Santos *et al*., 2007). The variability of the phenology found within this genus (see below) might be linked to the observed outcome.

Thaumetopoea life cycles

The pine processionary moth is a well-known defoliator occurring in the Mediterranean countries. It is considered as an important forest pest due to the damage caused by the larvae feeding on needles of several *Pinus* species, and a pest of public concern because the larvae from the 3rd instar onwards possess urticating hairs that cause severe allergic reactions to people and animals (Vega *et al*., 2004). The life stages and main aspects of the pest are illustrated in Fig. 1.

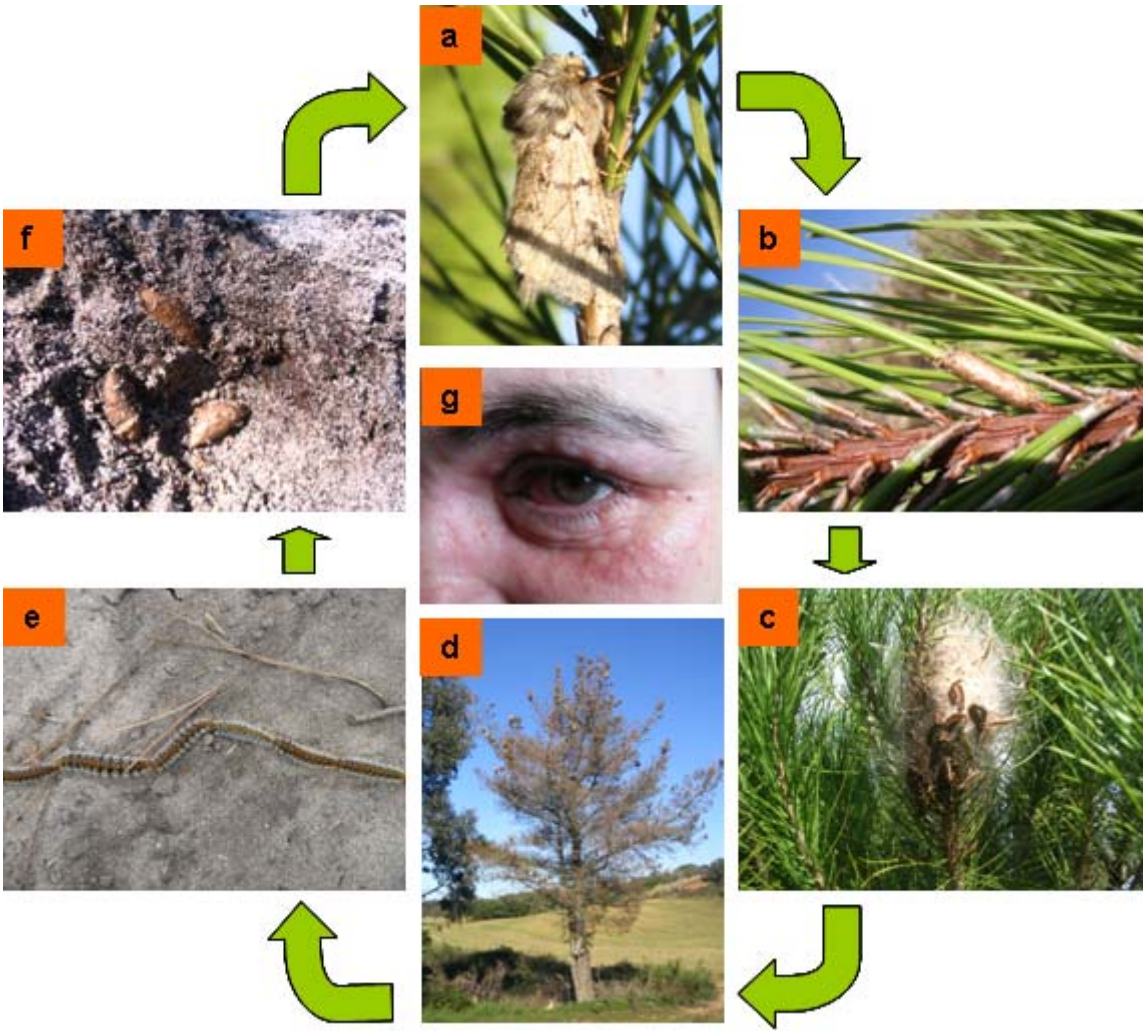

Figure 1 - Life stages and main aspects of the species. a - adult; b – egg batch; c – nest with larvae; d - defoliation; e – procession of larvae to pupate in the soil ; f – pupae; g – allergy caused by urticating hairs

In Portugal, only this species is associated with pines, although other species of the same genus also attack other pines, other conifers and even broad-leaved species (Agenjo, 1941) in Europe and in the Mediterranean basin.

Outbreaks and subsequent severe defoliations cause a decrease in radial growth and, therefore, important economic losses (Kulman, 1971; Carus, 2004; Kanat *et al*., 2005; Gatto *et al*., 2009; Arnaldo *et al*., 2010; Jacquet *et al*., 2012), as well as ecological impacts such as an increased susceptibility to other pests, like scolytids (Coleoptera, Scolytidae), *Pissodes castaneus* (DeGeer) (Col., Curculionidae), the pine weevil, or the *Dioryctria sylvestrella* (Ratz.), Lep., Pyralidae, the new pine knot-horn (Kulman, 1971; Ferreira & Ferreira, 1990; Markalas, 1998; Hódar *et al*., 2003; Kanat *et al*., 2005; Arnaldo *et al*., 2010).

Species of the genus *Thaumetopoea* can be grouped according to the existence of diapause in the egg or pupal stage, depending on their geographical and altitudinal distribution (Schmidt, 1989). In Europe, the genus is represented by the species *T. pityocampa* (winter pine processionary moth), occurring almost all over southern Europe; *T. pinivora* (Treitschke) (summer processionary) is distributed in West Sweden, Denmark, Northern Germany, France, Spain and Romania; *T. processionea* (L.) (oak processionary) whose distribution ranges from Finland and Scandinavia to the Iberian and Apennines Peninsula and from England to the Black Sea; and *T. solitaria* (Freyer.) (pistacia processionary) found in western former Yugoslavia, northern Greece, Bulgaria, European Turkey and Israel (Douma-Petridou, 1989). In the Mediterranean basin there are also other species such as *T. bonjeani* (Powell) (Cedar processionary), distributed in North Africa (Morocco, and Algeria); *T. herculeana* (Rambur) found in Spain, North Africa and Palestine on different bushes such as Cystus and Geranium; *T. wilkinsoni* (Tams), distributed in the Near East; and *T. jordana* (Staudinger), occurring in the arid zones of Israel, Jordan and Saudi Arabia (Schmidt, 1989). Although the cited author reports the occurrence of *T. herculeana* in Portugal, made by Rougeot & Viette in 1983 (see (Schmidt, 1989), and even though the author refers that biological data of this species is rare there are no records in the national forestry bibliography of the occurrence of this species.

According to Schmidt (1989) and Douma-Petridou (1989), three types of life cycles can be distinguished for the genus *Thaumetopoea*:

1. A biannual cycle, found in *T. pinivora*, in which the adults emerge in the summer months and egg laying takes place immediately. An egg diapause occurs during the winter and the larvae hatch in April–May. Larval development lasts about three months, followed by pupation. The pupae overwinter and the adults appear after a pupal diapause, in July-August of the coming year.

2. An annual cycle with an egg diapause in the winter, found in *T. processionea*, *T. bonjeani*, *T. solitaria* and *T. herculeana*, in which the eggs are deposited in the autumn and the young larvae hatch in April-May of the coming year. The larval development lasts about three months, followed by pupation. About a month later, the adults emerge and copulation and egg laying take place immediately.

3. An annual cycle with spring pupal diapause, found in *T. pityocampa*, *T. wilkinsoni* and *T. jordana*. The eggs are laid in the summer or fall, depending on the latitude and altitude. At sea level and southern areas adult flight and egg-laying takes place later in the year than in mountainous areas or northern regions. Climatic factors can modulate the timing of reproduction, with later adult emergences observed in warmer climates, and earlier emergences observed along higher latitudinal and altitudinal ranges (Démolin, 1969; Huchon & Démolin, 1970). The young larvae hatch after about 5-6 weeks and grow quickly reaching the 3^{rd}-4^{th} instar before wintertime. Except the larvae of *T. jordana*, which do not spin nests, the larvae of all other species spin silk nests. Larvae from early instars spin rather loose nests, while later instars spin very dense nests to keep temperature more efficiently. Depending on the winter temperature and altitude, the larval period lasts between three and eight months without a diapause. In mountainous regions the larval period is prolonged due to the long winters, during which the larvae feed less and grow more slowly. In most cases the larval period ends in March-April of the coming year. Larvae then migrate into the soil for pupation, forming long processions. After a pupal diapause of 3 to 5 months, the adults emerge, copulate and deposit the eggs in batches within less than 12 hours.

The spatial distribution of *Thaumetopoea* species is shown in Fig 2.

Figure 2 - Distribution of the main species of *Thaumetopoea* in the Mediterranean basin. Countries in white colour are those of occurrence of *T. pityocampa* (Adapted from: http://www.daapv.unipd.it/promoth/biology.htm).

The distribution of species in general, and insects in particular, as well as biological cycles can be related to climatic factors such as temperature, and insolation depending on the latitude and altitude (Powell & Logan, 2005; Visser & Both, 2005; Parmesan, 2006; Visser *et al*., 2010). Temperature is the most basic controller of seasonality in poikilothermic organisms (Zaslavski, 1988; Bale *et al*., 2002) and a large body of literature exists relating the effects of temperature to developmental rates and phenology (Powell *et al*., 2000). This is obviously true for processionary moths, and according to Démolin (1969), temperatures below –7ºC are considered lethal for single larvae, although there is some variation, depending on the number and stage of the larvae, and temperatures above 32ºC lead to a quick 100% mortality for young larvae (L1 and L2). Winter nests are true solar radiators that larvae use according to their needs of heat (Breuer *et al*., 1989; Breuer & Devkota, 1990; Breuer, 1997; Jones *et al*., 2006). The photoperiod is another factor affecting the behaviour of the larvae, as well as the daily thermal amplitudes. In the northern latitudes, winter temperatures commonly fall below lethal limits and photoperiod is too short, *T. pityocampa* no longer occurs, and *T. pinivora* is the species best adapted to this climate,

with a summer larval development while egg and pupal diapause allow to overcome winter harshness.

Nevertheless, different phenologies have been reported within *T. pityocampa*. For example, since about 40 years ago, a population of pine processionary moth with a distinct phenology from from that classically described has been studied in Banya, Bulgaria (Tsankov *et al*., 1996). According to these authors, the adults emerge from the soil between the end of June and July and start egg-laying immediately after copulation. The larvae hatch in the second half of July and the first half of August. Larval development is very fast and processions of 5th instar larvae can be observed as early as mid-October, migrating into the soil for pupation. Larvae remain in a pre-pupal stage until March-April of the coming year, and only then diapause occurs. Pupation can last until July, or can be prolonged for as long as 1-3 years. Populations of *T. pityocampa* with phenology similar to the one expected for the species are found in other regions of Bulgaria, and intensive research was developed since then for both populations. Natural enemies were found to be the same (Tsankov *et al*., 1996) and no unknown species of natural enemies were found for the abnormal population, which indicates that both populations belong to the same, or a very close species.

Leiria T. pityocampa summer population

In August of 1997 an abnormal defoliation of pine trees, and an exceptionally high number of nests and larvae of what appeared to be the pine processionary moth *Thaumetopoea pityocampa* Den. & Schiff. (Lepidoptera, Notodontidae), in advanced stages of development were observed in Mata Nacional de Leiria (MNL).

The situation was considered abnormal, since in August/September the pine processionary moth (PPM) should have been in the egg stage, and trees should be recovering from the previous winter defoliation. In Portugal, the cycle of *T. pityocampa* corresponds to what is described for the rest of Europe, although the 5th instar larvae procession to the soil in January, February or March, depending on the latitude and host species (personal observations).

This population with a distinct phenology was discovered in the Southern region of Mata Nacional de Leiria (MNL), by personal observation of Professor Maria Rosa Paiva, FCT, Universidade Nova de Lisboa. At the time, large nests of fully grown larvae in the final stage of development were observed, in an outbreak situation. Shortly afterwards, FCT-UNL and ISA-UTL teams started studying this unusual population of PPM which has been observed in high densities in the same area, expanding southwards and northwards (Pimentel, 2004; Santos, 2004; Pimentel *et al.*, 2006) for about a decade, while over the last five years much lower densities have been observed (Author, personal observations). The population with shifted phenology was named "summer population", or "SP", and the normal phenology populations were named "winter populations", or "WP", in relation to the respective timing of the larval stage, which is the most conspicuous. Pine processionary moth occurs naturally throughout the stands of MNL, in relatively low densities, varying with the age of the pines, with a life cycle similar to the populations on the rest of Portugal.

The summer population has a life cycle with larvae developing between mid June and late September/early October, followed by pupation in the soil that lasts until May, when the flight period of the adults starts. The adults emerge during May and June, when oviposition takes place. Larvae start hatching around mid June, and a new cycle begins (Pimentel, 2004; Pimentel *et al*., 2006) (Fig. 3).

The ecology of the two populations shares similar aspects, including natural enemies, namely the parasitoid complex, predators such as birds and other insects, and fungi of the genus *Beauveria*. Pheromone traps used to capture SP adults were baited with the same synthetic pheromone used for the winter populations, attracting SP males as documented by Pimentel *et al*. (2004; 2006) and Santos *et al*. (2004; 2007).

At first, several hypotheses were proposed to explain the origin of this population. Perhaps an introduction from a distant area where *T. pityocampa* could have another cycle, the consequence of a hybridization event with a related species of the same genus, or it could be a cryptic species.

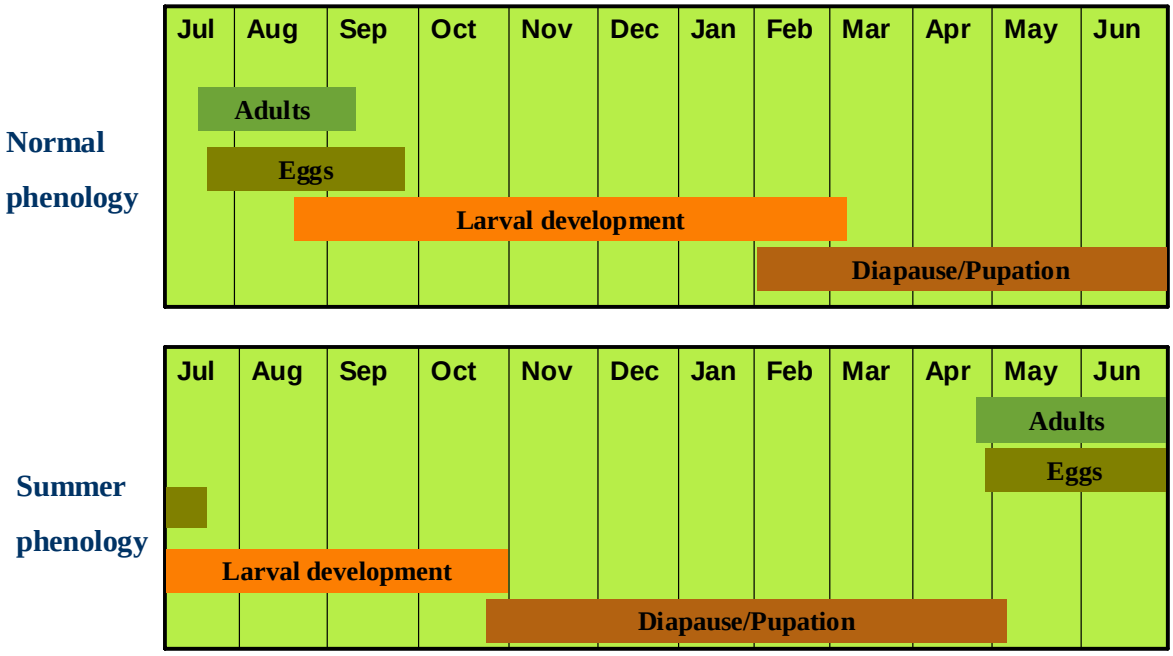

Figure 3 - Phenologies of the two populations: WP and SP.

The first results from the genetic studies based on mitochondrial and nuclear sequences revealed that the SP belongs to the same species as the surrounding WPs (*T. pityocampa*) and that it is not a cryptic species. These markers showed no differentiation between SP and Leiria WP, suggesting that the phenological shift is fairly recent (Santos et al., 2007). Conversely, microsatellite markers revealed a strong differentiation between SP and WP, with signs of founder event in the SP. However, these first conclusions should be considered with caution, as these preliminary data were based on a single sampling season, using a limited number of individuals genotyped with 5 microsatellite markers, and a reduced number of individuals sequenced for mitochondrial DNA (5) and ITS1 (2), which could be insufficient to reveal rare alleles or hybridization events.

These results thus suggested that the SP was recently established by a reduced number of individuals with early adult emergence and consecutive rapid larval development. In the field, monitoring of the adult flight period revealed reproductive isolation, a fair indicator that differentiation was ongoing and speciation could occur. Nevertheless, more thorough information was needed both from the genetic differentiation and the reproductive isolation to demonstrate that we are facing a rarely documented case of allochronic differentiation. The

question of possible ecological differentiation was also open. The study case will further offer a model and a unique opportunity to study this evolutionary mechanism *in situ.*

Leiria forest

Mediterranean climate is characterized by hot, dry summer and warm, wet winters, and pines are prominent components of the Mediterranean basin flora, covering 25% of all forest area, having invaded it 3,5 million years ago (Ribeiro, 2001). These conifers reveal a remarkable ecological plasticity and are known to prevail in areas with adverse climatic and edaphic conditions as well as in first succession stages (Tapias *et al.*, 2004). Pines have a high colonizing ability and play a central role in vegetation dynamics in the Mediterranean region, explained by a high genetic variability and/or phenotypic plasticity (Tapias *et al*., 2004).

The forest area in Portugal is mostly (about 33%) occupied by maritime pine *Pinus pinaster* Aitton and stone pine *P. pinea* L. (AFN, 2010a). According to fossil, charcoal and palynological analysis, *P. pinaster* has been present in Portugal since 55000 years ago, and was probably able to survive the last glaciations in sheltered areas at low altitude close to the Atlantic ocean, but the occupied area would be much smaller (Figueiral, 1995; González-Martínez *et al*., 2004). Pine processionary moth is present throughout Portugal, including urban areas, wherever pines are present. All pine species are attacked, both autochthonous and exotic species. Outbreaks are frequent in some areas, mostly in young pine monocultures.

Mata Nacional de Leiria is located in the centre seashore zone of Portugal, located 150 km North of Lisbon, between latitudes 39º42’N and 39º53'N and longitudes 8º55’'W and 9º02'W, with a maximum altitude of 136m. The pines occupy an area of about 11 023 ha, of which 10 828 ha are covered with *P. pinaster* stands (Fig. 4).

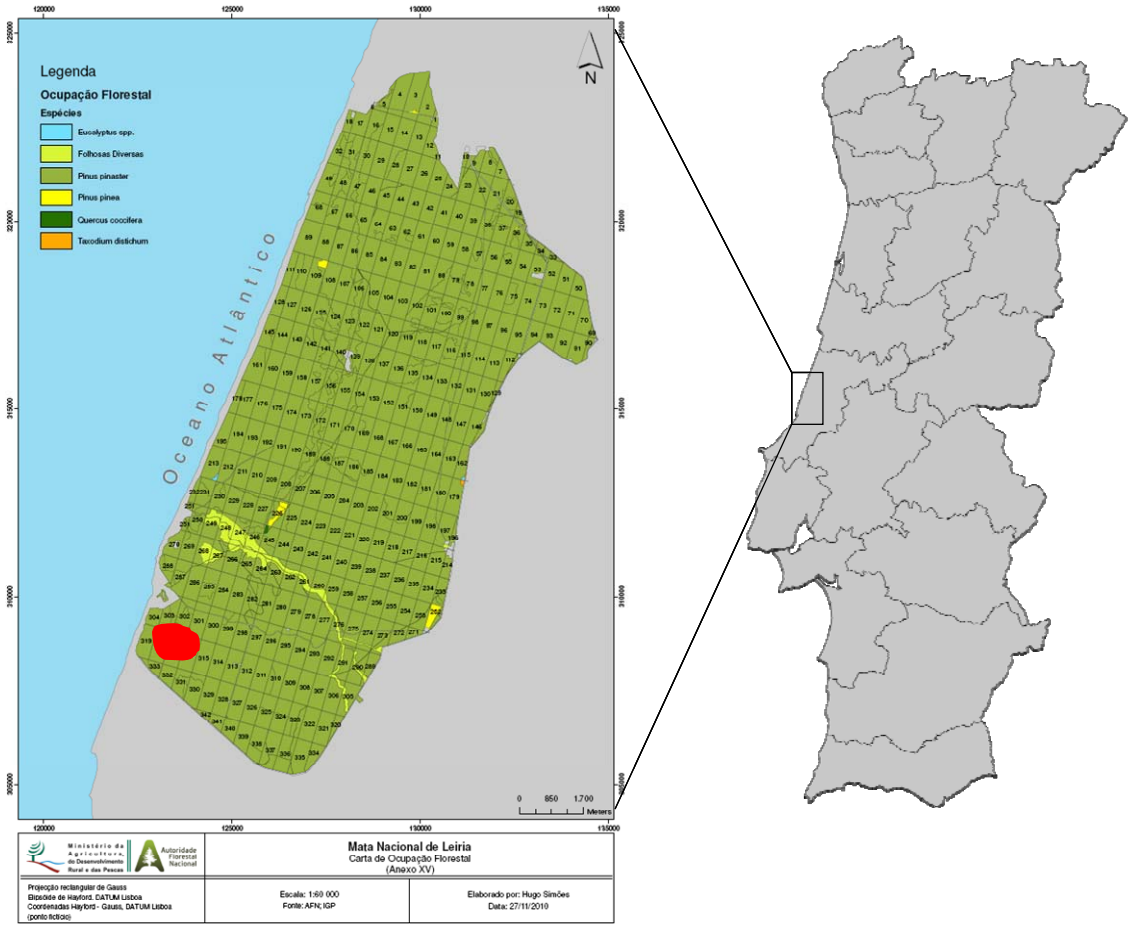

Figure 4 - Map of Portugal with the location of the Leiria Pine Wood (right) and detailed soil occupation in 2010. The area where SP was discovered in 1997 is highlighted in red. (adapted from (AFN, 2010b).

The climate, according to the Köppen classification is mesothermal, or humid temperate climate, with a dry, long and cool summer (Marques, 2010).

The geological substratum is largely dominated by Quaternary formations, which include mainly sand dunes and Pliocene sediments consisting of dunes and sand dunes (Zbyszewski & Torre de Assunção, 1965b, a). The relief is planed to undulating, depending on the expression of the dunes, which form three main strands which roughly follow the direction of the coast. The soils of "Mata Nacional de Leiria" are predominantly regosols and Podzolized Soils (Cardoso, 1974; Marques, 2010).

In addition to *P. pinaster* the most abundant species in the understory are *Calluna vulgaris* (L.) Hull, heather; *Halimium calycinum* (L.) K. Koch, yellow rock rose; *Cistus salvifolius* L., Sage leaf rock rose; *Pteridium aquilinum* (L.) Kuhn, Braken; *Ulex europaeus* subsp. *latebracteatus* (L.), Gorse.

Pinewoods have been managed in this area at least since the XIII century, having a protective function in stabilising the dunes, protecting agricultural areas and producing timber. The areas close to the ocean undergo natural regeneration, resulting in low tree densities and low growth rates. King D. Dinis (1261–1325) extended the already existing area aiming to the production of high quality timber and established the first management rules. Other pine species also occur in this area, but *P. pinaster* is by far the most important one.

Leiria is thus a very particular and unique area, as it has been occupied with *P. pinaster* for more than five centuries and is characterized by a very particular climate which might have influenced this unique case of allochronic speciation studied in this work.

Objectives

Overall we aim at understanding the evolutionary and ecological mechanism involved on the differentiation of the two populations reproductively isolated, also as tool to predict its future evolution and expansion. The specific objectives of this study are:

1. To confirm, genetically, the occurrence of a founder effect in the SP and to analyse the genetic structure of the two sympatric populations by: i) determining the stability in time, of the shifted phenology by monitoring the complete flight periods of both populations over several years, using pheromone traps; ii) exploring for signs of hybridization by sequencing CO1 mitochondrial marker, to assure that no divergent haplotypes occur in the SP; and iii) testing the stability over years of the genetic structure of both SP and WP.

2. To estimate the genetic divergence between the sympatric populations and compare the genetic structure with other populations.

3. Since the two sympatric populations have similar life stages in different seasons, subjected to different ecological constrains, to explore ecological adaptations and differences between the populations, namely i) adaptation to temperature of the larval stages; ii) Morphological differences in the egg stage.

4. To test the capacity of the sympatric populations to actually hybridize, by artificially manipulating adult emergence, and to assess the viability of the hybrids.

5. To analyse and compare the phenology patterns of the parental populations and of the hybrid descendants.

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2. INCIPIENT ALLOCHRONIC SPECIATION IN THE PINE PROCESSIONARY MOTH (*Thaumetopoea pityocampa*, LEPIDOPTERA, NOTODONTIDAE)

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2. H. Santos, C. Burban, J. Rousselet, J.P. Rossi, M. Branco & C. Kerdelhué (2011) Incipient allochronic speciation in the pine processionary moth (*Thaumetopoea pityocampa*, Lepidoptera, Notodontidae). Journal of Evolutionary Biology, 24, 146–158. doi: 10.1111/j.1420-9101.2010.02147.x

Abstract

A plausible case of allochronic differentiation, where barrier to gene flow is primarily due to a phenological shift, was recently discovered in Portugal for the pine processionary moth *Thaumetopoea pityocampa*. Previous results suggested that the observed "summer population" (SP) originated from the sympatric winter population (WP). Our objectives were to finely analyse these patterns and test their stability in time, through field monitoring and genetic analyses of larvae and adults across different years. Reproductive activity never overlapped between SP and WP. Microsatellites showed a clear differentiation of the SP, consistent with a strong reduction of gene flow due to the phenological shift. Assignment tests suggested that some individuals shift from the SP to the WP phenology, causing some hybridization. We discuss these patterns and their maintenance over time. This could be a first stage of allochronic speciation and SP should be considered as a distinct phenological race.

Keywords: Allochronic differentiation; sympatric speciation; phenology; pine processionary moth; microsatellites

1. Introduction

The process by which species diverge into different strains that become reproductively isolated and evolutionarily independent is well-known and documented for numerous cases of spatial isolation known as allopatric speciation (e.g Johannesson, 2001; Turelli *et al.*, 2001). Speciation in sympatry is yet a controversial subject, and the proposed models require conditions that are difficult to obtain in natural populations (Bolnick & Fitzpatrick, 2007). Nevertheless, a few case studies have shown that it could be more common than originally thought (Bush & Smith, 1998). Sympatric speciation occurs when reproductive isolation is observed while the different populations still exist in the same area. Host or habitat specialization is one of the most documented processes leading to sympatric isolation and speciation, mostly in fishes and phytophagous insects (e.g Berlocher & Feder, 2002; Dres & Mallet, 2002; Blair *et al*., 2005; Feder *et al*., 2005; Peccoud *et al*., 2009).

Yet, sympatric speciation can also occur because of separation of populations by breeding time, a process known as allochronic speciation (Alexander & Bigelow, 1960). Such a situation occurs when populations reproduce in different years as observed in periodical Cicadas (Marshall & Cooley, 2000; Simon *et al*., 2000; Cooley *et al*., 2001; Ritchie, 2001), in different seasons of the year (Alexander & Bigelow, 1960); (Friesen *et al*., 2007), or even in different times of the day (Miyatake *et al*., 2002). In many insect groups, the adult stage is short-lived, thus small differences in phenology can prevent adults occurring in the same area from mating. Reproductive isolation can occur if there are differential non-overlapping reproductive seasons and if these phenologies are heritable. In some cases, phenological differentiations have been documented in phytophagous insects as an adaptation to the phenology of different hosts (Bolnick & Fitzpatrick, 2007). Very few cases of allochronic speciation due to phenological isolation *per se* (without host plant diversification) have been studied so far (Abbot & Withgott, 2004; Santos et al., 2007; Yamamoto & Sota, 2009) probably due to its scarcity.

The pine processionary moth (PPM) (*Thaumetopoea pityocampa* Den. & Schiff., Lepidoptera, Notodontidae), is a well-known defoliator occurring in the Mediterranean countries (Kerdelhué *et al*., 2009). It is considered as an important forest pest due to the damages caused

by the larvae feeding on needles of several *Pinus* species, and a pest of public concern because of the larvae's urticating hairs that cause severe allergic reactions to people and animals (Vega *et al.*, 2004). The larvae are gregarious, inhabit silk nests and pupate below ground. Adults mostly emerge during summer, reproduce and die within a few days (Démolin, 1969). In Portugal, at low altitudes where the populations studied in the present paper are located, adults classically reproduce between the end of July and September and larval development occurs during fall and winter (Figure 1A). Pupation starts in February or March, with an obligate pupal diapause, until new adults emerge in summer. A facultative prolonged diapause can occur (Huchon & Démolin, 1970), leading to the emergence of adults one or several years later, still in the end of summer.

An exceptional phenological shift from a winter larval development to a summer larval development was observed in one single site in Portugal, in the National Forest of Leiria (Pimentel et al., 2006) which raised ecological and evolutionary questions. Locally, individuals with a normal life cycle (adult emergence in summer and larval development in winter) co-occur with individuals that have a shifted life-cycle, with adults emerging in spring and larvae developing during summer (Figure 1B). Larvae from this population are exposed to different conditions from those developing in winter, as climate, host phenological stage, and active natural enemies differ between seasons. This abnormal population has a limited geographical range, restricted to a 10 x 50 km forest of pure even-aged maritime pine stands (*Pinus pinaster* Ait.) named the "Mata Nacional de Leiria" (MNL), the management of which dates back to the XIVth century. Throughout this paper, the unique shifted population will be referred to as the "summer population" (SP) while all other populations, that exhibit the common and widespread phenology will be called "winter populations" (WP). The specific Leiria winter population, which is in sympatry with the SP, will be referred to as "Leiria WP".

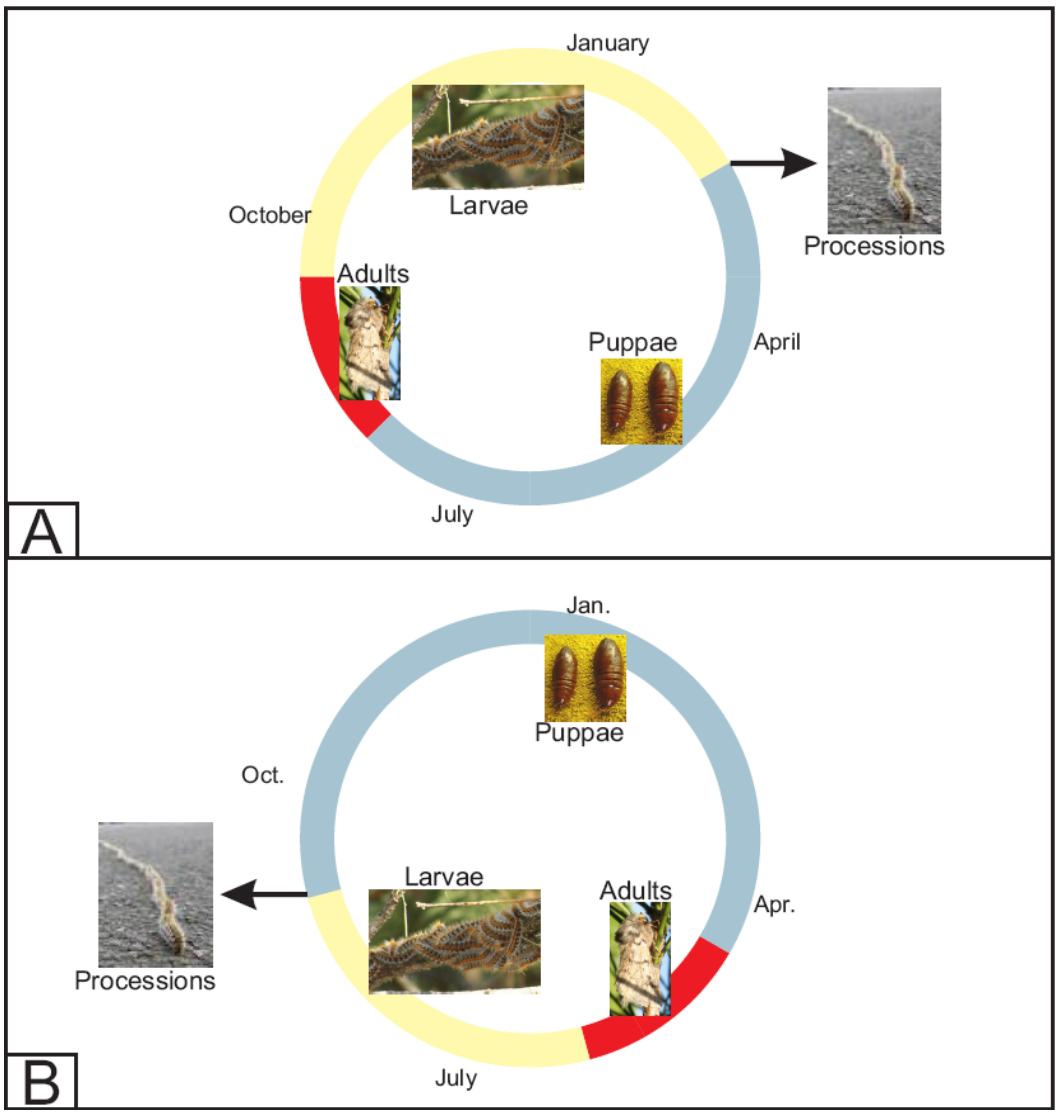

Figure 1 – Biological cycle of *Thaumetopoea pityocampa* in Portugal. A. Normal cycle observed at low altitude including in Leiria (i.e. for Leiria WP), with a winter larval development. B. Shifted cycle observed solely for Leiria SP, with a summer larval development.

Previous results suggested that allochronic differentiation was on-going, as a direct consequence of the phenological shift. Based on mitochondrial and nuclear sequences, an earlier study hypothesized that the SP belongs to the same species as the surrounding WPs (*T. pityocampa*) and that it is not a cryptic species (Santos *et al.*, 2007). These genetic markers showed no differentiation between SP and Leiria WP, suggesting that the phenological shift is fairly recent. Conversely, 5 microsatellite markers revealed a strong differentiation between SP

and WP, with signs of founder event in the SP. These first results thus suggested that the SP was recently established by a reduced number of individuals with early sexual reproduction and consecutive rapid larval development. However, these first conclusions should be considered with caution, as these preliminary data were based on one single sampling season per population, using a limited number of larvae, and only global, Fst-based analyses of differentiation. Moreover, no adults were then included, and the genetic origins of the males trapped during monitoring were not analysed. In the present study, we gathered new data (larvae, males and females collected during successive years and analysed with 6 microsatellite markers) and proceeded to detailed analyses of individual genetic assignment and of population differentiation to (1) precisely determine the phenology of *T. pityocampa* in Leiria, by monitoring the complete flight periods of both SP and WP populations in several years through pheromone traps; (2) test the stability in time of the genetic structure of both SP and WP, by genotyping with 6 microsatellite loci individual larvae and adults sampled over three different years; and (3) check if all individuals, including trapped adults, could be correctly assigned to their reference populations.

2. Material and Methods

2.1 Monitoring of males flight period

In order to assess whether mating disruption between both populations could be hypothesized, we tested whether the adult activity of the Leiria WP and SP could eventually overlap by monitoring the flight of adult males from early May to the end of September in 2005, 2007 and 2008, covering the whole known flight period in Leiria. To provide reference data for the normal phenology in Portugal, another site (Apostiça) located ca. 100 kms south of Leiria, at similar altitude and longitude, was monitored in 2005 and 2008. Funnel traps baited with synthetic PPM pheromone dispensers (10515/BFL072, AgriSense™) were hung on trees at a reachable height. Twelve to fourteen traps were distributed all over the known area of the summer population at 1–5 km intervals, and 5 to 12 traps were placed in Apostiça in 20 to 50 m

intervals. Pheromone dispensers were replaced every six weeks. All funnels had a DDVP (2,2-dichlorovinyl dimethyl phosphate, BIOSANI) tab inside, to act as a killing agent. Traps were assessed weekly from May to July and weekly or every two weeks from August to September.

2.2 Sampling and DNA extraction

Samples of *T. pityocampa* collected in Leiria in 2002, 2005, 2006, 2007 and 2008 were used to complete the original dataset of larvae collected during 2003-2004 (Santos *et al.*, 2007), and consisted of larvae, adult males and adult females from both summer and winter populations. Additional larvae were collected in September 2002 and 2005 for the winter population, and July 2006 and 2007 for the summer population. Males and females were collected in 2007 and 2008. Concerning the populations from Leiria, each individual was coded *a priori* as belonging either to the SP or the WP based on phenotypic data (date of collect for the larvae, and date of emergence or trapping for the adults). Samples consisted of 30 (15 in 2002) first instar larvae per population and year, each originating from different nests to prevent sampling siblings. First-instar larvae collected in May or June were phenotypically coded as SP, while those collected in September and October were coded as WP. Males were captured by pheromone trapping as described above (individuals captured before the end of June were *a priori* coded as SP, and individuals trapped after the end of July as WP). Females were obtained from laboratory rearing of pupae and larvae collected in the field in 2008 from both SP and WP (based on the date of emergence of adults in the lab) from Leiria and from a winter population in Apostiça, located south of Lisbon. Sampling details are given in Table 1. All samples were collected in maritime pine stands (*Pinus pinaster*) and immediately stored in 95% ethanol. DNA was extracted from the whole body of PPM larvae and from thoracic muscles of adults, using the GenElute mammalian Genomic DNA miniprep kit (Sigma) and eluted in 200 µl of buffer.

Table 1: Sampling localities and date of collection of the 341 genotyped individuals of *Thaumetopoea pityocampa,* and number of genotyped individuals per population. Data from Santos *et al*. (2007) are indicated.

Population Code	Population type	Development stage	Country	Locality	Geographical coordinates	Year of collect	# Genotyped individuals	Genotypes for MS-*Thpit*1, 3, 4, 5 & 6 from:	Genotypes for MS-*Thpit*2 from:
LSP03	Summer	L1 larvae	Portugal	Leiria	39º47' N 8º 58' W	2003	29	Santos *et al*. 2007	Present paper
LSP06	Summer	L1 larvae	Portugal	Leiria	39º47' N 8º 58' W	2006	30	Present paper	Present paper
LSP07	Summer	L1 larvae	Portugal	Leiria	39º47' N 8º 58' W	2007	30	Present paper	Present paper
LSP07-M	Summer	Adult males	Portugal	Leiria	39º47' N 8º 58' W	2007	16	Present paper	Present paper
LSP08-F	Summer	Adult females	Portugal	Leiria	39º47' N 8º 58' W	2008	19	Present paper	Present paper
LWP02	Winter	L1 larvae	Portugal	Leiria	39º47' N 8º 58' W	2002	15	Present paper	Present paper
LWP04	Winter	L1 larvae	Portugal	Leiria	39º47' N 8º 58' W	2004	36	Santos *et al*. 2007	Present paper
LWP05	Winter	L1 larvae	Portugal	Leiria	39º47' N 8º 58' W	2005	30	Present paper	Present paper
LWP07-M	Winter	Adult males	Portugal	Leiria	39º47' N 8º 58' W	2007	15	Present paper	Present paper
LWP08-F	Winter	Adult females	Portugal	Leiria	39º47' N 8º 58' W	2008	2	Present paper	Present paper

Apostiça-F	Winter	Adult females	Portugal	Apostiça	38º34' N 9º 07' W	2008	17	Present paper	Present paper
Alcácer	Winter	L1 larvae	Portugal	Alcácer	38º 23’ N 8º 31’ W	2002	31	Santos *et al*. 2007	Present paper
Viseu	Winter	L1 larvae	Portugal	Viseu	40º 40’ N 7º 54’ W	2002	24	Santos *et al*. 2007	Present paper
Guadarrama	Winter	L1 larvae	Spain	Guadarrama	40°41' N 4°02' W	2002	24	Santos *et al*. 2007	Present paper
Granada	Winter	L1 larvae	Spain	Granada	37º 47' N 3º 46' W	2002	23	Santos *et al*. 2007	Present paper

2.3 Microsatellite genotyping

Six microsatellite loci were used to genotype the newly sampled individuals. Five of these loci, namely MS-*Thpit*1, MS-*Thpit*2, MS-*Thpit*3, MS-*Thpit*4 and MS-*Thpit*5, are described in Rousselet *et al*. (2004), and MS-*Thpit*6 is described in Santos *et al*. (2007). Moreover, MS-*Thpit*2 was used to genotype the individuals studied in this latter reference, which were previously analysed using only 5 microsatellite markers. Fluorescent PCR products were run and detected on an ABI 3730 automatic sequencer and product sizes were determined using the Genemapper v4.0 software (Applied Biosystems). We used 17 positive controls from our previous work to ensure that alleles were correctly identified, and this new data set was merged with all data from Santos et al. (2007).

For MS-*Thpit*2, exceptionally long alleles were found in the SP (see results). Four individuals having long alleles were amplified and cloned using the Strataclone PCR cloning kit (Stratagene). Eight to ten clones were sequenced per individual using the BigDye terminator v3 cycle sequencing kit (AppliedBiosystems) and carried out with an ABI 3100 automatic sequencer. This procedure ensured that both alleles were sequenced for each individual, and permitted to check whether the long sizes of these alleles were actually due to a higher number of repetition of the microsatellite motif, or to an insertion.

2.4 Data analyses

File format conversions were all done using CONVERT (Glaubitz, 2004). **A**llelic richness and frequencies, as well as observed and expected heterozygosities were calculated for each locus using GENETIX v. 4.04 (Belkhir *et al.*, 1996-2004). Histograms of allelic frequencies were built using R (R Development Core Team, 2008). Hardy-Weinberg equilibrium (HWE) was tested using ARLEQUIN 3.11 (Excoffier *et al.*, 2005) for each locus and population, with a significance level of 95%, using 1000 permutations steps and 100,000 steps in the Markov chain. Linkage-disequilibrium (LD) was tested in each population for all pairs of loci with 10,000 permutations using ARLEQUIN. Null allele frequencies were estimated for each locus using the

Expectation Maximization (EM) algorithm of Demster *et al*. (1977) as performed in the FREENA package (Chapuis & Estoup, 2007).

Analyses were performed using first instar larvae, and then repeated with all sampled individuals (i.e. including males and females). Larval populations were considered as "reference populations", both because their location can be clearly identified and because only embryonic mortality has occurred. On the other hand, adult populations may have been strongly affected by selection, and the individuals may have flown and been trapped far from their locality of origin.

Two loci, namely MS-*Thpit*2 and MS-*Thpit*6, showed significant departures from HWE (see results). Subsequently, all analyses were first performed using all loci and then repeated excluding these two loci to check for consistency of results.

Population genetic structure**:** Population structure was first analysed using pairwise F_{ST} (Weir & Cockerham, 1984) estimated using the ENA correction implemented in FREENA to correct for the positive bias induced by the presence of null alleles (Chapuis & Estoup, 2007). The 95% confidence intervals of corrected pairwise Fst values were obtained by bootstrapping 1000 times over loci.

The Principal Component Analysis (PCA) was then used to analyse the microsatellite data set. One advantage of the PCA is that it is free from strong assumptions about an evolutionary model (e.g. HWE), which is particularly valuable to analyse poorly known biological systems (Patterson *et al.*, 2006; Jombart, 2008; Jombart *et al.*, 2009). PCA was complemented by a statistical procedure called the between-class test, to assess the between-populations divergences. The procedure compares the observed between-class inertia (i.e. variance) to the corresponding values stemming from randomizations (Dolédec & Chessel, 1987; Manly, 1997). Both PCA and randomization tests were performed using the R packages adegenet (Jombart, 2008) and ade4 (Chessel *et al.*, 2004). A PCA was first performed with the larvae sampled from the SP (all years), from Leiria WP (all years) and from the four additional sites sampled in Portugal and Spain. The between-population test was done using 1000 randomizations. A complementary analysis was then performed using all sampled individuals, including larvae, males and females.

Individual assignments: We used larvae and adults sampled at different dates in Leiria to assign individuals to clusters based on their multilocus genotype using a Bayesian inference method implemented in STRUCTURE 2.3.3 (Pritchard *et al.*, 2000). We fixed the number K of clusters to 2, as the objective was to test whether individuals could be correctly assigned to either the SP or the Leiria WP. We used 100,000 burn-in steps followed by 100,000 MCMC simulation steps with a model allowing admixture. To assess the consistency of results, we performed 25 independent runs and carefully compared the obtained individual Q-matrices. The results were graphically displayed using DISTRUCT 1.1 (Rosenberg, 2004).

We also performed assignment tests using GENECLASS2 (Piry *et al.*, 2004) to specifically test if reference populations of larvae could be assigned or excluded as origin of the trapped adults based on their genotypes. We used four reference populations containing only larvae, namely Leiria SP (all years), Leiria WP (all years), Portugal (Alcacer and Viseu) and Spain (Guadarrama and Granada), and assigned all males and females trapped in Leiria and in the near-by site of Apostiça. We used the Bayesian method described in Rannala and Mountain (1997). For each individual, the score of assignment to each reference population was computed as defined in Piry *et al*. (2004)

Test of a founder effect: the allelic richness was compared between Leiria SP and Leiria WP to test if it was significantly reduced in the SP. The test was done for larvae from LWP02, LWP04 and LWP05 for Leiria WP, and LSP03, LSP06 and LSP07 for Leiria SP (see Table 1 for details). We used the two sample Wilcoxon test (also known as the Mann-Whitney test) to compare the median of the average allelic richness (i.e., the allelic richness divided by the sample size) in WP vs. SP. We used the function wilcox.test from the R software (R Development Core Team, 2008). The procedure was done for each locus separately, and with all loci together. Due to the peculiar results obtained with MS-*Thpit*2, the test was repeated with 5 loci only.

3. Results

3.1 Monitoring of males flight period

In Leiria, for the 3 years of study, the males started to emerge between the 3rd and 12th of May, and were mostly trapped between the end of May and the end of June. No male was captured between the end of June and the end of July, and then a small number of males were captured in August and September. In all years of monitoring, the shortest interval between male captures (i.e., the time during which no males were trapped) in Leiria was 24 days (in 2007) and the longest was 41 days (2005). In the reference winter population of Apostiça the flight season occurred between August and the end of September in all years (Figure 2).

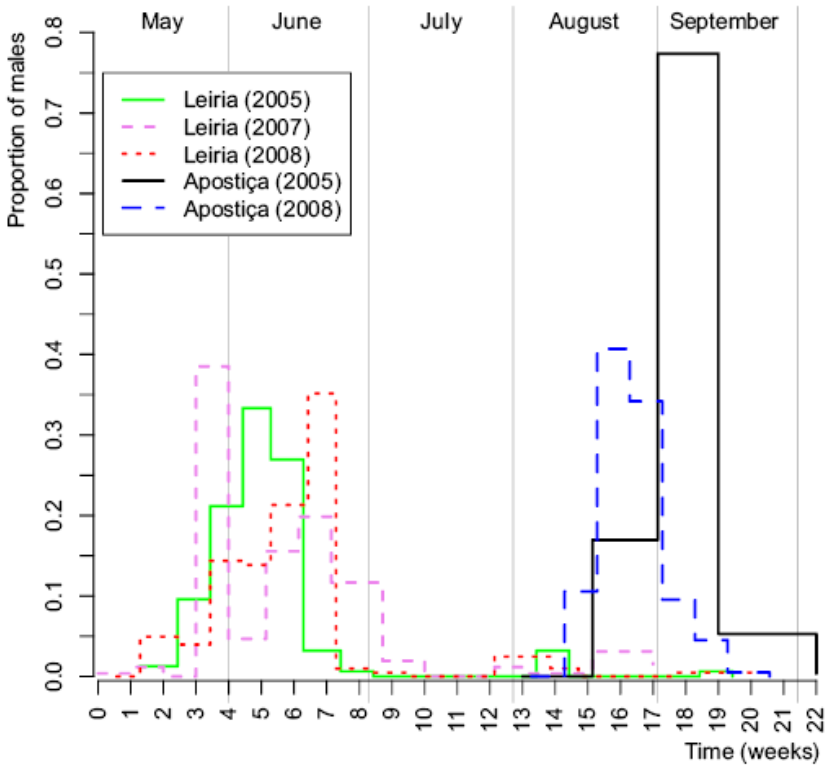

Figure 2 – Males caught with pheromone traps from May to September in Leiria (2005, 2007 and 2008) and Apostiça (2005 and 2008). For each date, data are expressed as the proportion of males caught by site and by year.

3.2 Microsatellite results

The new data obtained here were merged with the data available from Santos *et al*. (2007) to obtain a final data set of 341 individuals genotyped with 6 markers.

The total number of alleles per locus varied from 8 in locus MS-*Thpit*5 to 40 in locus MS-*Thpit*6. The total number of alleles per population ranged from 27 to 32 in Leiria SP, from 33 to 47 for Leiria WP, and from 53 to 57 in the other Portuguese and Spanish WPs (Table 2) except for the females collected in Apostiça (Table 1).

Table 2: Number of individuals (N), observed (Ho) and expected (He) heterozygosities, percentage of null alleles (%NA) per locus estimated using FREENA, and total number of alleles found in each *T. pityocampa* population. Population codes are given in Table 1.

Population Code	N	Ho	He	%NA MS-*ThPit*1	%NA MS-*ThPit*2	%NA MS-*ThPit*3	%NA MS-*ThPit*4	%NA MS-*ThPit*5	%NA MS-*ThPit*6	Total number of alleles
LSP03	29	0.48	0.51	0.000	0.041	0.000	0.102	0.000	0.181	30
LSP06	30	0.46	0.48	0.000	0.045	0.000	0.011	0.000	0.121	29
LSP07	30	0.46	0.49	0.000	0.055	0.000	0.000	0.000	0.227	32
LSP07-M	16	0.47	0.48	0.000	0.000	0.000	0.000	0.021	0.000	27
LSP08-F	19	0.44	0.52	0.000	0.000	0.002	0.000	0.076	0.354	27
LWP02	15	0.52	0.63	0.000	0.219	0.010	0.000	0.089	0.116	33
LWP04	36	0.52	0.61	0.000	0.150	0.000	0.000	0.067	0.261	47
LWP05	30	0.51	0.62	0.027	0.148	0.015	0.004	0.000	0.279	43
LWP07-M	15	0.58	0.70	0.000	0.172	0.101	0.000	0.000	0.000	35
Apostiça-F	17	0.44	0.55	0.097	0.000	0.000	0.037	0.000	0.435	23
Alcácer	31	0.60	0.75	0.006	0.105	0.019	0.051	0.000	0.291	50
Viseu	24	0.68	0.76	0.023	0.049	0.009	0.000	0.000	0.120	56
Guadarrama	24	0.64	0.73	0.000	0.065	0.000	0.000	0.000	0.234	57
Granada	23	0.69	0.81	0.000	0.128	0.000	0.000	0.053	0.262	53

Distribution of allelic frequencies in Leiria WP and SP is shown for all markers in Figure 3, and the detailed allelic frequencies are given in Supplementary Information (Table S1). Except for locus MS-*Thpit*2, all the alleles found in Leiria SP were also present in Leiria WP while the opposite was not true. In several cases, the allelic frequencies of the SP were distorted compared to the frequencies of the Portuguese WPs. For example, MS-*Thpit*1 is almost fixed for allele 165 (96%) in the SP, whereas this allele is never above 61% in any Portuguese WP. Likewise, for locus MS-*Thpit*3 the allele 239 has very low frequencies in WP (values between 2% and 8%) while it is the most frequent in SP, with values ranging between 46% and 67% depending on the

year of sampling. Results obtained from locus MS-*Thpit*2 were completely different, as it was the single locus for which the number of alleles in the SP was higher than in the WP. Longer alleles were found only in the SP for this locus. In all winter populations (including Leiria), allele sizes ranged from 143 to 163 bp whereas alleles up to 183 bp were found in the SP. The "long alleles" (>165 bp) were found for each sampled year of the SP in similar proportions, and the corresponding chromatograms were unambiguous. Cloning and sequencing indicated that these alleles showed an increase in the number of repetitions, consistent with the observed size, and no mutation in the flanking regions or interruptions due to insertions in the microsatellite motif. Consequently, we can confidently assume that these long alleles and the peculiar results obtained for marker MS-*Thpit*2 are not an artefact.

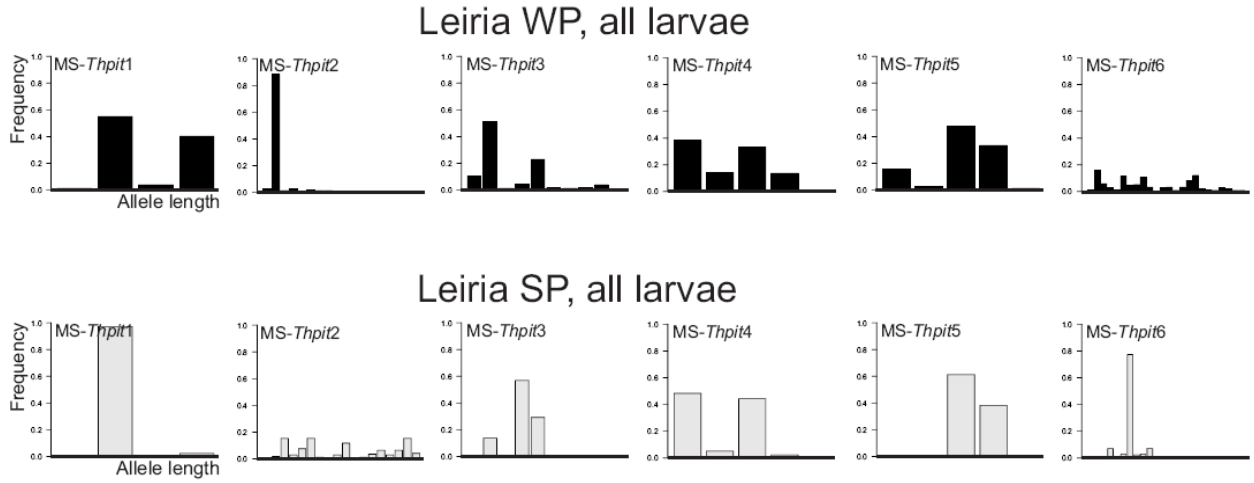

Figure 3 - Allelic frequencies found for larvae sampled in Leiria WP and Leiria SP for the 6 studied microsatellite markers. In each population, the frequencies were calculated with individual larvae sampled from all years.

All populations were in HWE for MS-*Thpit*1, MS-*Thpit*3, MS-*Thpit*4 and MS-*Thpit*5, except population LSP03 for MS-*Thpit*5 and Granada for MS-*Thpit*1 and MS-*Thpit*4. On the contrary, no population was in HWE for MS-*Thpit*6 except the male samples (LSP07-M and LWP07-M). Finally, most populations were not in HWE for MS-*Thpit*2 (all populations but LSP07, LPS07-M, LSP08F, Apostiça-F and Viseu). Consistently, using the EM algorithm available in FREENA, these two loci (MS-*Thpit2* & MS-*Thpit*6) were estimated to have high

proportions of null alleles in several populations (Table 2). Interestingly, all populations except the male samples (LSP07-M and LWP07-M) had over 10% of null alleles for MS-*Thpit*6, and the two female samples (LSP08-F and Apostiça-F) even reached 35 and 43%. Moreover, all females were homozygous for this locus. Concerning MS-*Thpit*2, the estimations were over 10% of null alleles for all the samples of the Leiria WP (LWP02, LWP04, LWP05 and LWP07-M), as well as for Alcácer and Granada. No pairs of loci were in LD in more than 2 populations, except for the pairs MS-*Thpit*1 – MS-*Thpit*6 and MS-*Thpit*2 – MS-*Thpit*3 that were in LD in 3 populations. Hence, the microsatellite loci used were considered independent.

3.3 Population genetic structure

The matrix of pairwise F_{ST} obtained with the ENA correction for the presence of null alleles (Chapuis & Estoup, 2007) is given in Table 3. These indices were significant between any pair of populations except when comparing different years of the SP (Pairwise $F_{ST} < 0.011$, whatever the development stage – larvae, males or females) or different years of Leiria WP when considering only larvae (pairwise $F_{ST} < 0.012$). Interestingly, the pairwise differentiation indices were slightly higher when comparing males Leiria WP to larvae from the same locality (F_{ST} ranging between 0.021 and 0.042, this latter value being significant). The differentiation between any year of SP *vs*. any year of Leiria WP was always above 18.0% and reached 26.6% except when comparing the Leiria WP males to any Leiria SP samples (Fst were in this case between 0.075 and 0.123), indicating that sympatric SP and WP are significantly and strongly differentiated. F_{ST} values between Leiria SP and the populations sampled in other Iberian localities were comprised between 13.2% with Alcácer, and 32.4% with Apostiça. The pairwise F_{ST} obtained between pairs of geographically distant winter populations were significant but lower than those obtained between any SP and any WP. Differentiation indices between Leiria WP and any other WP were comprised between 1.6% (with Viseu) and 15.1% (with Guadarrama). When omitting MS-*Thpit*2 and MS-*Thpit*6 from the analyses, we obtained very similar F_{ST} estimates (Table S2, Supporting information).

Table 3: Pairwise F_{ST} matrix obtained using all microsatellite loci after applying the ENA correction for null alleles using FREENA; significant indices are shown in bold

	LSP03	LSP06	LSP07	LSP07-M	LSP08-F	LWP02	LWP04	LWP05	LWP07-M	Apostiça-F	Alcácer	Viseu	Guadarrama
LSP06	0.010												
LSP07	0.003	0.003											
LSP07-M	0.010	-0.001	-0.001										
LSP08-F	-0.004	0.014	-0.002	0.011									
LWP02	**0.223**	**0.266**	**0.261**	**0.264**	**0.217**								
LWP04	**0.201**	**0.239**	**0.237**	**0.243**	**0.194**	0.006							
LWP05	**0.188**	**0.225**	**0.224**	**0.232**	**0.180**	0.012	-0.007						
LWP07-M	**0.086**	**0.120**	**0.120**	**0.123**	**0.075**	**0.042**	0.028	0.021					
Apostiça-F	**0.276**	**0.318**	**0.312**	**0.324**	**0.271**	0.038	**0.036**	**0.026**	**0.083**				
Alcácer	**0.153**	**0.181**	**0.173**	**0.190**	**0.132**	**0.087**	**0.093**	**0.077**	**0.046**	**0.110**			
Viseu	**0.161**	**0.201**	**0.190**	**0.200**	**0.143**	**0.048**	**0.048**	**0.038**	**0.016**	**0.056**	**0.026**		
Guadarrama	**0.182**	**0.212**	**0.204**	**0.222**	**0.167**	**0.151**	**0.143**	**0.119**	**0.096**	**0.139**	**0.034**	**0.047**	
Granada	**0.232**	**0.260**	**0.252**	**0.249**	**0.213**	**0.113**	**0.126**	**0.120**	**0.100**	**0.145**	**0.071**	**0.061**	**0.075**

A PCA was run including Leiria SP and WP as well as geographically distant WPs. The two first axes explained 19.7% and 10.1% of the total inertia, respectively. Axis one separated Leiria SP from all other WPs, and axis 2 separated the different WPs (Figure 4).

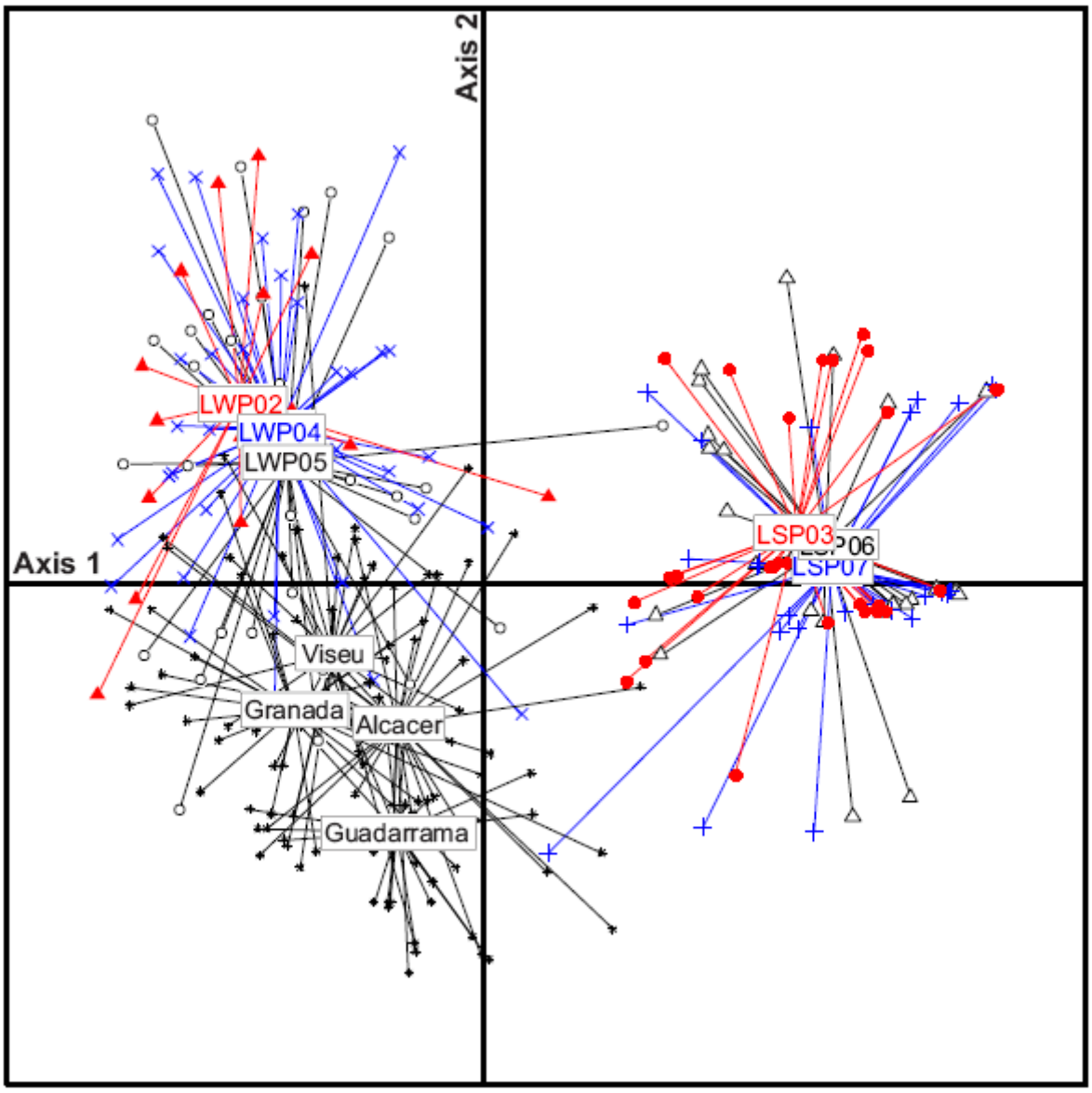

Figure 4 - Typology of PPM larvae obtained by PCA of all 6 microsatellite data, projection of individuals upon the plane defined by components 1-2. Lines link individuals to the corresponding population (codes are given in Table 1). The analysis included all larvae sampled in Leiria (WP and SP) in different years, and individuals sampled in different localities of Spain and Portugal.

The between-class test was significant ($p<0.001$) revealing strong genetic differences. Similar results were obtained without MS-*Thpit*2 and MS-*Thpit*6. When the PCA was performed

using all individuals (i.e. larvae, males and females), the axes explained 19.13% and 8.65% of the total inertia, respectively, and the between-class test was also highly significant. Consistently, males and females sampled from the Leiria SP clearly grouped with the larvae of the same population, but only 12 males trapped in Leiria WP (i.e., males trapped in Leiria between the end of July and September) grouped with the corresponding larvae, while 3 clustered with the larvae of the SP (Figure S1, Supporting information). The females sampled from Apostiça were very close to the Leiria WP.

3.4 Individual assignments

Larvae and adults from Leiria, sampled in different seasons and years, were analysed using the Bayesian method implemented in STRUCTURE 2.3.3 with a number of clusters K = 2, to check if the genetic assignment matches the *a priori* phenotypic populations (SP and WP). The individual genetic assignments were very similar across the 25 runs performed. The individual Q-matrix of membership coefficients is graphically displayed in Figure 5.

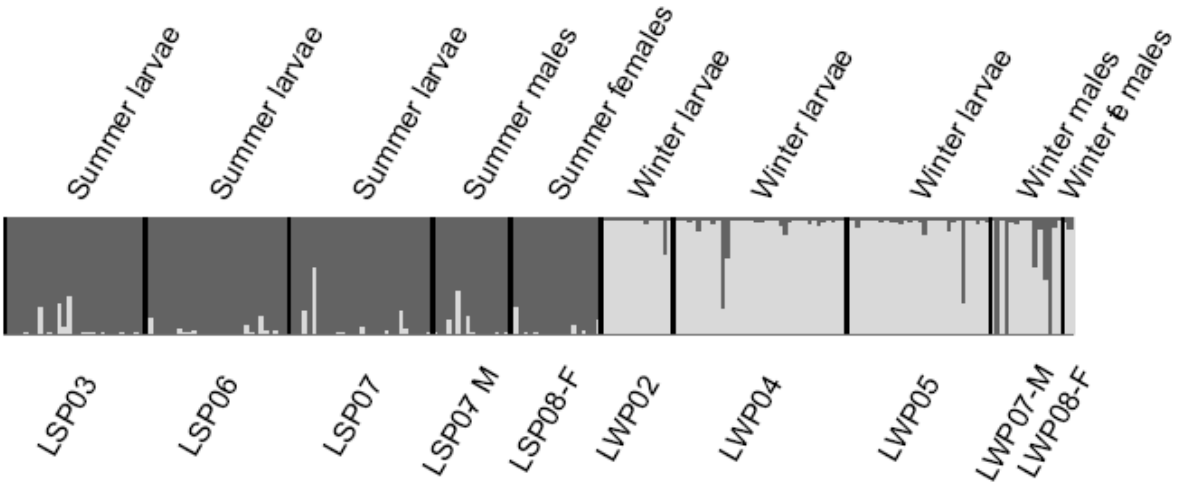

Figure 5 – Graphic representation of the individual Q-matrix (mean over 25 runs) obtained for K = 2 and 222 individuals (larvae, males and females) sampled from Leiria in different years and different seasons.

The two main genetic clusters obtained actually mostly corresponded to the "phenotypic" SP and WP. For clarity, the two clusters will be called "genetic SP" and "genetic WP", while the phenotypic groups (determined *a priori* on the basis of phenological phenotypes, i.e. on the date

of hatching for larvae and the date of emergence for males and females) will be called "phenotypic SP" and "phenotypic WP". For 196 of the 222 individuals (88%), the genetic cluster fully corresponded to the phenotypic group, with membership coefficients Q over 0.90 (this value reaching 205 individuals (92%) for a threshold of 0.80 for the membership coefficient). For 10 individuals, the genetic cluster corresponded to the phenotypic group but with Q membership coefficients comprised between 0.59 and 0.80. For one "phenotypically WP" male, the genetic assignation was intermediate (membership coefficients of 0.49 for WP and 0.51 for SP). On the other hand, for three larvae, the genetic and the phenotypic groups did not match, but membership coefficients were moderate (one "phenotypically SP" larva was genetically assigned to WP with Q = 0.58, and two "phenotypically WP" larvae were genetically assigned to SP with Q = 0.76 and Q = 0.73, respectively). Finally, it is worth noting that 3 males trapped during the normal flight season of the WP (thus "phenotypically WP") were assigned to the "genetic SP" with very high coefficients (0.99). Those 3 males were also clustered with the SP individuals in the PCA.

The results obtained with GENECLASS2 to assign adults to the reference larval populations were largely consistent with those obtained with STRUCTURE. All SP males and females were genetically assigned to the larval populations of Leiria SP with assignment scores (as defined in Piry *et al.*, 2004 p. 538) close to 100, except for one female that was preferentially assigned to the other Portuguese populations, with a score of 87. For comparison, using STRUCTURE, this particular female was assigned to the "genetic SP" with a membership coefficient of 0.87. Concerning the males trapped during the WP flight period, three individuals were assigned to the SP larvae with maximal scores (100); consistently, these individuals were also assigned to the "genetic SP" using STRUCTURE. All other WP males were assigned to either the Leiria WP larval population, or to near-by Portuguese populations. The same was true for all the females collected in Apostiça.

3.5 Test of a recent founder effect

Statistical analysis (Wilcoxon test) of allelic richness locus by locus revealed that Leiria WP had a significantly higher allelic richness for loci MS-*Thpit*1 (p=0.036), MS-*Thpit*3 (p=0.05), MS-*Thpit*5 (p=0.05) and MS-*Thpit*6 (p=0.05), but that no significant statistical differences were

observed between Leiria SP and Leiria WP for locus MS-*Thpit*4 (p=0.25). On the contrary, locus MS-*Thpit*2 had a significantly higher allelic richness in the SP than in the sympatric WP (p=0.05). Concerning analyses done with all loci together, SP had a significantly reduced global allelic richness compared to WP (p=0.05). The results were similar when MS-*Thpit*2 was excluded from the analysis (p=0.05).

4. Discussion

4.1 Some preliminary considerations

Our study is based on a comprehensive sampling of larvae and adults of *T. pityocampa*, mostly from Leiria, that was genotyped using 6 microsatellite markers. Yet, two of these markers had significant proportions of null alleles, and showed deviations from Hardy-Weinberg Equilibrium. Even though the results were robust enough to be consistent even when omitting these two markers, it is important to understand how reliable they are. Concerning MS-*Thpit*6, our data show that male samples are in HWE for this locus and have a proportion of null alleles estimated to zero. On the contrary, the female samples are not in equilibrium, with a high proportion of null alleles. Moreover, all females (i.e. 38 individuals) are systematically homozygous for this locus in spite of the high number of alleles found, and the high corresponding expected heterozygosity. The most plausible explanation is thus that this microsatellite marker is located on the sexual Z chromosome (in Lepidoptera, females are heterogametic (Z - W) while males are Z – Z).

On the other hand, the results concerning MS-*Thpit*2 are puzzling, as it is the single locus showing a set of alleles that were unique from the Leiria SP. These "long" alleles (see Figure 3) were not detected elsewhere, neither in Leiria WP, nor in the other Iberian populations. They were also not observed in a previous study based on six populations scattered all over France (Kerdelhué *et al.*, 2006). Yet, the estimations of null allele frequencies suggest that many null alleles occur for this locus in all Leiria WP samples, while it is not the case for the SP, nor for most other Iberian populations. Similarly, a low proportion of null alleles were observed in the French populations mentioned above (not shown). We can thus confidently consider that the genotypes observed in Leiria SP are reliable, and that the long alleles are actually absent from

other regions of Portugal, Spain and France. Yet, when discussing and interpreting our data, we have to keep in mind that the results obtained for MS-*Thpit*2 in Leiria WP are biased due to the presence of null alleles and amplification problems.

4.2 Phenology suggests complete reproductive isolation…

In 4 years of monitoring, we always observed a bimodal curve of adult emergence in Leiria, whereas typical curves of male trapping in the PPM are unimodal (Démolin, 1969b). A period of 26 to 42 days without any male catch was observed each year around July. One peak of male activity is observed from the beginning of May to early July (with a maximum reached in June, see Figure 2), and the other from the end of July to late September, which is consistent with earlier data (Pimentel *et al*., 2006a). The second peak corresponds to the normal period of male activity for the PPM in Portugal at low altitudes, as is exemplified by the data we obtained in Apostiça as well as previously published data (Barrento *et al.*, 2008). The most plausible hypothesis is thus that the first peak corresponds to the emergence of the SP adults, and the second one to the WP adults (but see below). The low number of male captures within the normal emergence periods of WP males in Leiria is also consistent with field observations showing that the population size of Leiria WP is low (H. Santos & M. Branco, ISA, Lisbon, pers. obs.). Whether this is related to the existence of the SP, or due to other environmental conditions or to natural changes in population dynamics remains an open question. Our data strongly suggest that a time lag of ca. one month occurs each year between the latest emergences of the SP and the earliest emergences of the sympatric WP. As adults do not feed, and live up to a maximum of 3-4 days (Démolin, 1969b, H. Santos, ISA, Lisbon, pers. obs.), we could conclude that SP and WP individuals cannot mate, and that sexual reproduction between the two sympatric populations is limited, or even non-existent. Phenology effectively leads to pre-zygotic isolation.

4.3 … and genetic data show a clear differentiation, but with a limited flow of individuals from SP to WP.

Data on male captures alone do not allow ruling out the hypothesis that genetic exchange still occurs between populations. After larval development, an obligate diapause occurs during the pupation stage, and diapause termination determines the dates of adult emergence (Huchon & Démolin, 1970b). Gene flow between both populations could thus occur via individuals shifting

from one phenology to the other through the mechanisms underlying adult emergence. In other words, some "winter larvae" could emerge as "summer adults" after pupal diapause, and vice-versa. If this was the case, genetic evidence of gene flow between populations should be found, and we could expect the within-population genetic structure to vary in time as a consequence of gene flow.

Yet, the genetic data we obtained for different years showed both a clear and extreme differentiation between SP and WP, and the stability in time of the genetic structure observed in Leiria. The pairwise F_{ST} matrix, the PCA and the Bayesian analyses run with STRUCTURE all show that the SP is significantly differentiated from all studied WP. Individuals sampled in different years from the SP clearly fall in the same genetic cluster (yet with the exception of one larva from LSP07), and a similar pattern was mostly observed for Leiria WP (with only two mis-assigned larvae). Differentiation between different localities of WP is significant but always lower than the SP vs. WP indices. The results were robust, as similar conclusions were found even when omitting the two microsatellite markers MS-*Thpit*2 and MS-*Thpit*6. Thus, our main conclusion is that the Leiria SP forms a very distinct genetic cluster, which is highly stable in time. The most plausible force responsible for the genetic isolation is allochronic differentiation, as the SP and the local WP apparently differ only by their timing of emergence and sexual reproduction. As a consequence, we suggest that the Leiria SP could now be considered as a highly differentiated "phenological race", in the same manner that host races have been proposed for phytophagous or parasitoid insects lineages using different hosts (e.g. Dres & Mallet, 2002; Blair *et al*., 2005; Stireman *et al*., 2005; Stireman *et al*., 2006; Peccoud *et al*., 2009). Individuals from the SP now experience unique selective pressures as compared to the other PPM populations, such as temperature and hygrometry during metamorphosis, embryogenesis and the first stages of larval development, quality of consumed foliage and natural enemies. Natural selection is thus likely to favour the evolution of adaptations to these peculiar conditions, which could lead to reinforcement of the differentiation. Allochronic speciation has been hypothesized in a few cases, as a mechanism to explain a past speciation event between fully separated taxa (Cooley *et al*., 2001; Ritchie, 2001; Abbot & Withgott, 2004; Danley *et al*., 2007). We discovered here an exceptional case of on-going allochronic differentiation that could be seen as a first stage of an incipient sympatric speciation without host shift.

Interestingly though, the Bayesian analysis of population structure and the assignment tests done with the adults clearly show that a limited amount of gene flow can occur between the SP and the WP. Among the 222 individuals, three larvae were assigned to the wrong genetic cluster compared to their phenotypic group, with membership coefficients Q close to 0.75, two males *a priori* "phenotypically" WP had intermediate Q (0.48 and 0. 59 for the WP, respectively), and three males trapped during the WP normal flight period where even genetically assigned to the SP with Q = 0.99. These results will need to be confirmed by increasing the number of males analysed, but they strongly suggest that a proportion of individuals can shift from the SP to the normal "winter" phenology. We hypothesize that individuals from the SP can occasionally experience a longer diapause, and emerge as adults in July or August rather than April or May. They can then reproduce with WP individuals, causing a limited amount of gene flow between populations. This is probably a rare event, as it was never observed in laboratory conditions (MB & HS, pers. obs.). This would explain both the existence of "phenologically WP but genetically SP" males, and the occurrence of larvae that are identified by STRUCTURE as genetic hybrids. The opposite shift (from a normal WP phenology to the SP) is apparently very rare, as the SP adults were all unambiguously assigned to the SP genetic cluster, and a large majority of the SP larvae were correctly assigned to the SP genetic cluster. It is thus puzzling that a strong genetic differentiation is maintained between SP and WP in spite of flows of individuals. This result could suggest that shifting individuals and hybrids could be counter-selected due to a mal-adaptation to environmental conditions, and hence do not significantly contribute to the next generations. Laboratory experiments will also be necessary to test whether SP and WP individuals can cross and give fertile offspring.

4.4 Local origin of the summer population?

Different scenarios can be proposed to explain the existence of a PPM population with a shifted phenology. Based on previous sequencing and genotyping data, Santos *et al.* (2007) favoured a scenario involving a recent local origin of the summer population from a sudden phenological "mutation" and a bottleneck effect. The new data presented here are mostly consistent with this hypothesis. Except for MS-*Thpit*2, we observed a lower number of alleles in the SP than in the WP for all sampling seasons, with all alleles found in SP also present in Leiria WP (but not the opposite), and a distortion of the allelic frequencies. However, the data obtained

for MS-*Thpit*2 are contradictory, as its alleles are more numerous in the SP than in the WP, and many alleles present in the SP were not found elsewhere. In particular, we found a whole group of long-sized alleles that were not observed even in the other Iberian populations. It is very unlikely that the new alleles appeared locally after the differentiation of the SP from mutation alone, both because it would be possible only if the summer population was founded thousands of years ago (if we consider that the average mutation rate for microsatellite markers is ca. 10^{-3}, Goldstein & Pollock, 1997) and remained undetected, which is not likely for this well-known and conspicuous species; and because no new alleles were detected for the other five loci used. These data can be consistent with the scenario of local phenological shift and foundation of the summer population from few individuals of the sympatric WP if one supposes that all the long alleles detected in SP were rare but present in the WP, but remain undetected due to sampling or technical biases (null alleles, see "technical considerations" above). More makers will be necessary in the future, even though developing microsatellites in Lepidoptera is not straightforward (Zhang, 2004), to determine if MS-*Thpit*2 is the only locus showing this kind of pattern, and if all other loci confirm the scenario involving a founder effect.

Our results clearly show the occurrence of a sympatric allochronic differentiation process. The most plausible scenario is that a phenologically shifted population has recently been founded by individuals originating from the local genetic pool, and that very limited gene flow now occurs between both populations. This could be the first stage of allochronic speciation, which is a rarely documented process. The SP can be seen as a "phenological race", exposed to different ecological pressures and constraints that could cause further divergence and maintain the genetic differentiation. Ecological consequences and factors potentially limiting the expansion of the SP should now be analysed. Moreover, laboratory rearing and experimental crosses will be necessary to better understand the differentiation (and possibly the taxonomic status) of both populations.

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SUPPORTING INFORMATION:

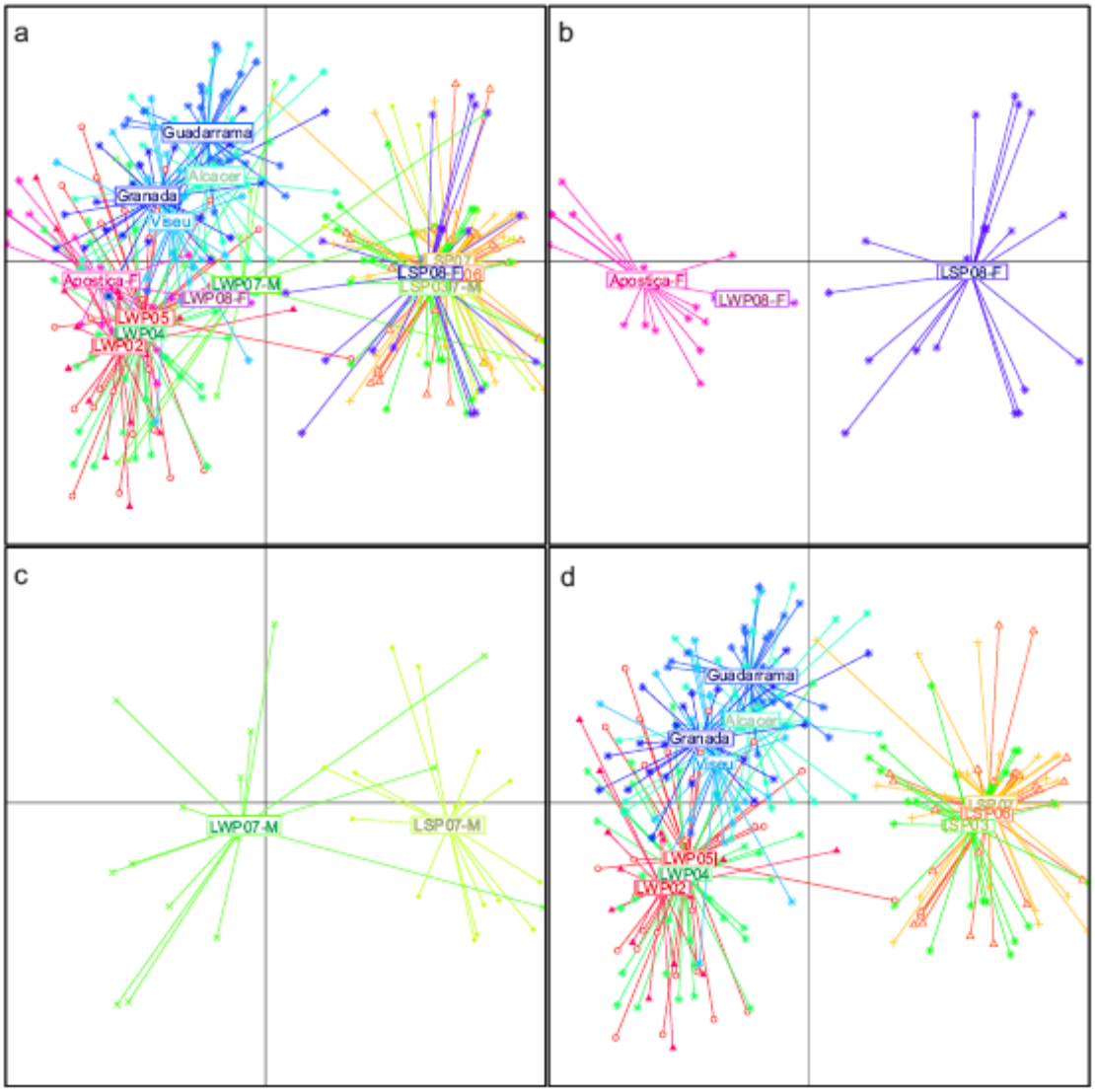

Figure S1 - Typology of all PPM individuals (larvae, males and females) obtained by PCA of all six microsatellite data, projection of individuals upon the plane defined by components 1–2. Lines link individuals to the corresponding population (codes are given in Table 1). (a) All individuals. (b) Only the dots corresponding to adult females are shown. (c) Only the dots corresponding to adult males are shown. (d) Only the dots corresponding to larvae are shown.

Table S1: Allelic frequencies for each microsatellite locus and each population

Locus	Allele	LSP03	LSP06	LSP07	LSP07-M	LSP08-F	LWP02	LWP04	LWP05	LWP07-M	LWP08-F	Apostiça-F	Alcacer	Viseu	Guadar-rama	Granada
MS-*Thpit*1	163	0	0.02	0	0	0	0	0.01	0.02	0	0	0	0.05	0	0.06	0.17
	165	0.97	0.98	0.97	0.94	0.97	0.43	0.56	0.60	0.63	1.00	0.53	0.61	0.54	0.77	0.30
	167	0	0	0	0	0	0	0.03	0.07	0.07	0	0	0.13	0.15	0.06	0.22
	169	0.03	0	0.03	0.06	0.03	0.57	0.40	0.32	0.30	0	0.47	0.21	0.27	0.04	0.13
	171	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0.02
	173	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0.02	0
	175	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0
	179	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0
	181	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.09
	195	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.07
MS-*Thpit*2	135	0	0	0	0	0	0	0	0	0	0	0	0	0	0.06	0
	137	0	0	0	0	0	0	0	0	0	0	0	0	0	0.04	0.02
	139	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0
	141	0	0	0	0	0.03	0	0	0	0	0	0	0.04	0	0.04	0.04
	143	0	0	0	0	0	0	0.05	0.02	0	0	0.03	0.11	0.13	0.21	0.20
	145	0.02	0.04	0	0.07	0.03	0.83	0.91	0.88	0.39	0.75	0.94	0.30	0.39	0.27	0.41
	147	0.12	0.23	0.12	0.20	0.18	0.07	0	0	0.11	0	0	0.20	0.02	0.04	0.02
	149	0.03	0	0.05	0	0.13	0	0.03	0.03	0	0	0	0.20	0.17	0.04	0.04
	151	0	0	0	0	0	0	0	0	0	0	0.03	0.04	0	0.08	0.17
	153	0.09	0.10	0.05	0.10	0.13	0	0	0.02	0.22	0	0	0.13	0.07	0.02	0
	155	0.19	0.04	0.22	0.13	0.11	0.03	0	0.03	0	0.25	0	0	0.02	0.06	0.02
	157	0	0	0.03	0	0	0	0.02	0.02	0	0	0	0	0.07	0.06	0.07
	159	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0.02	0
	161	0	0	0.02	0.03	0	0.07	0	0	0	0	0	0	0.07	0	0
	163	0.02	0.06	0.02	0	0	0	0	0	0.06	0	0	0	0.02	0	0
	165	0.17	0.06	0.12	0.03	0.18	0	0	0	0	0	0	0	0	0	0
	167	0	0	0	0.07	0.03	0	0	0	0	0	0	0	0	0	0
	169	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0
	171	0	0.04	0	0.03	0	0	0	0	0	0	0	0	0	0	0
	173	0.03	0.04	0.03	0.10	0	0	0	0	0	0	0	0	0	0	0
	175	0.09	0.06	0.05	0.10	0.08	0	0	0	0.11	0	0	0	0	0.02	0
	177	0.05	0.02	0.02	0	0	0	0	0	0	0	0	0	0	0.02	0
	179	0.07	0.06	0.07	0.07	0.05	0	0	0	0	0	0	0	0	0	0
	181	0.05	0.27	0.15	0.07	0.05	0	0	0	0.11	0	0	0	0	0	0
	183	0.07	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0
MS-*Thpit*3	231	0	0	0	0	0	0.23	0.07	0.08	0.10	0	0.24	0.05	0.23	0.08	0.27
	233	0.24	0.08	0.09	0.09	0.22	0.47	0.56	0.48	0.50	0.25	0.47	0.23	0.40	0.13	0.18
	235	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0.10	0.18

	237	0	0	0	0	0	0	0.01	0.02	0	0	0	0	0.08	0	0.05
	239	0.47	0.57	0.68	0.69	0.58	0	0.06	0.05	0.17	0	0	0.08	0.02	0	0
	241	0	0	0	0	0	0	0	0	0.03	0	0	0.05	0.04	0.15	0.02
	243	0.29	0.35	0.23	0.19	0.19	0.20	0.19	0.28	0.17	0.75	0.21	0.26	0.10	0.29	0.02
	245	0	0	0	0	0	0.03	0.01	0.02	0	0	0	0.21	0.08	0.21	0.11
	247	0	0	0	0	0	0	0	0	0	0	0	0.10	0	0.04	0.02
	249	0	0	0	0	0	0	0	0	0.03	0	0	0	0	0	0
	251	0	0	0	0	0	0.03	0.01	0	0	0	0	0	0	0	0.11
	253	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0
	255	0	0	0	0.03	0	0	0.03	0.02	0	0	0	0	0	0	0
	257	0	0	0	0	0	0	0.04	0.05	0	0	0.09	0.02	0	0	0
	259	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0	0
	263	0	0	0	0	0	0.03	0.01	0	0	0	0	0	0	0	0
	273	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02
MS-*Thpit*4	161	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0
	165	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02
	167	0.52	0.45	0.48	0.53	0.53	0.30	0.36	0.45	0.43	0	0.50	0.19	0.29	0.31	0.26
	171	0.03	0.08	0.03	0.03	0	0.10	0.19	0.10	0.10	0	0	0	0.08	0	0.02
	173	0	0	0	0	0	0	0	0	0	0	0	0.16	0.06	0.08	0.09
	175	0.41	0.45	0.47	0.44	0.42	0.33	0.33	0.33	0.43	1.00	0.15	0.42	0.23	0.10	0.26
	177	0.03	0.02	0.02	0	0.05	0.27	0.10	0.12	0.03	0	0.26	0.23	0.13	0.08	0.02
	179	0	0	0	0	0	0	0	0	0	0	0.09	0	0.02	0.06	0.07
	181	0	0	0	0	0	0	0	0	0	0	0	0	0.06	0.10	0.20
	183	0	0	0	0	0	0	0	0	0	0	0	0	0.04	0.15	0
	185	0	0	0	0	0	0	0.01	0	0	0	0	0	0.02	0.10	0.02
	187	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0
	189	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02
	193	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0
	195	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02
MS-*Thpit*5	199	0	0	0	0	0	0.17	0.17	0.13	0.13	0.25	0	0.02	0.04	0	0.17
	201	0	0	0	0	0	0	0	0	0	0	0.06	0.23	0.08	0.31	0.39
	203	0	0.02	0	0.03	0	0	0.03	0.03	0	0	0	0	0	0	0.24
	205	0.64	0.62	0.58	0.63	0.61	0.57	0.47	0.43	0.43	0.50	0.32	0.16	0.27	0	0
	206	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0
	207	0.36	0.37	0.42	0.34	0.39	0.27	0.32	0.38	0.43	0.25	0.62	0.53	0.56	0.63	0.20
	209	0	0	0	0	0	0	0.01	0.02	0	0	0	0	0.04	0.04	0
	223	0	0	0	0	0	0	0	0	0	0	0	0.06	0	0	0
MS-*Thpit*6	138	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0
	146	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0.06	0
	148	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.27
	150	0	0	0	0	0	0	0.03	0	0	0	0	0.02	0.08	0.04	0.09
	152	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02	0.02

154	0	0	0	0	0.05	0	0	0	0	0	0	0.08	0.02	0.02	0.09
156	0	0	0	0	0	0.10	0.14	0.20	0.14	1.00	0.13	0.06	0.23	0.06	0.16
158	0	0	0	0	0	0	0.07	0.07	0	0	0	0.06	0	0.19	0.02
160	0.07	0.07	0.07	0.13	0.05	0.07	0	0.03	0	0	0	0	0	0.10	0
162	0	0	0	0	0	0.03	0	0.02	0.04	0	0	0.23	0	0.04	0
164	0.04	0	0.05	0	0	0.20	0.10	0.08	0	0	0.38	0.06	0	0.04	0.09
166	0.75	0.81	0.76	0.83	0.68	0	0.07	0.03	0.29	0	0	0.05	0.04	0	0.09
168	0	0.05	0.02	0	0.11	0	0.07	0.05	0.04	0	0.06	0	0.15	0.06	0
170	0.04	0.02	0.03	0	0.05	0.03	0.13	0.12	0.14	0	0.25	0.03	0.23	0.15	0.09
172	0	0	0	0	0	0	0	0	0.04	0	0	0.06	0.02	0.06	0.02
174	0.09	0.05	0.07	0	0.05	0	0.03	0.05	0	0	0	0.02	0.04	0.06	0.02
176	0.02	0	0	0	0	0	0	0	0	0	0	0	0.06	0	0
178	0	0	0	0.03	0	0	0.04	0.02	0.04	0	0	0.03	0.02	0	0
180	0	0	0	0	0	0.07	0.01	0.03	0.04	0	0	0	0.04	0	0
182	0	0	0	0	0	0	0	0.02	0.04	0	0.06	0.03	0	0	0.02
184	0	0	0	0	0	0.10	0.03	0	0	0	0	0.03	0	0	0
186	0	0	0	0	0	0.13	0.04	0.10	0.07	0	0	0.08	0.02	0	0
188	0	0	0	0	0	0.03	0.13	0.15	0.07	0	0	0.02	0.02	0.02	0
190	0	0	0	0	0	0.10	0	0	0	0	0	0.02	0	0.02	0
192	0	0	0	0	0	0	0	0	0	0	0	0.03	0	0	0
194	0	0	0	0	0	0	0	0	0	0	0.06	0	0	0	0
196	0	0	0	0	0	0	0	0	0.04	0	0	0.03	0	0	0
210	0	0	0	0	0	0	0.03	0	0	0	0	0	0	0	0
214	0	0	0	0	0	0	0	0	0.04	0	0	0	0	0	0
216	0	0	0	0	0	0	0.01	0	0	0	0	0	0	0.02	0
218	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0	0
220	0	0	0	0	0	0.03	0	0	0	0	0	0	0	0	0
222	0	0	0	0	0	0.03	0.03	0.02	0	0	0	0	0	0	0
224	0	0	0	0	0	0	0.03	0.02	0	0	0	0.02	0	0	0
226	0	0	0	0	0	0	0	0	0	0	0	0.02	0	0	0
228	0	0	0	0	0	0	0.01	0	0	0	0	0	0	0	0
230	0	0	0	0	0	0	0	0	0	0	0.06	0	0	0	0
236	0	0	0	0	0	0	0.01	0	0	0	0	0	0	0	0
240	0	0	0	0	0	0.03	0	0	0	0	0	0	0	0	0
242	0	0	0	0	0	0.03	0	0	0	0	0	0	0	0	0

Table S2: Pairwise F_{ST} matrix obtained using only 4 of the microsatellite loci, after applying the ENA correction for null alleles using FREENA; the excluded loci are MS-*Thpit*2 and MS-*Thpit*6. Confidence intervals were not estimated due to the low number of loci used.

	LSP03	LSP06	LSP07	LSP07-M	LSP08-F	LWP02	LWP04	LWP05	LWP07-M	Apostiça-F	Alcácer	Viseu	Guadarrama
LSP06	0.000												
LSP07	0.011	-0.004											
LSP07-M	0.006	-0.005	-0.018										
LSP08-F	-0.008	-0.001	-0.008	-0.017									
LWP02	0.190	0.237	0.255	0.225	0.206								
LWP04	0.127	0.173	0.187	0.167	0.142	0.005							
LWP05	0.100	0.144	0.161	0.144	0.115	0.015	-0.005						
LWP07-M	0.084	0.129	0.138	0.119	0.092	0.023	-0.005	-0.014					
Apostiça-F	0.189	0.241	0.251	0.232	0.201	0.036	0.041	0.022	0.033				
Alcácer	0.144	0.166	0.178	0.170	0.147	0.091	0.080	0.059	0.052	0.068			
Viseu	0.145	0.185	0.193	0.176	0.151	0.041	0.033	0.020	0.011	0.010	0.024		
Guadarrama	0.192	0.214	0.230	0.221	0.196	0.185	0.155	0.122	0.122	0.114	0.040	0.062	
Granada	0.257	0.278	0.289	0.255	0.248	0.132	0.136	0.129	0.122	0.137	0.087	0.077	0.103

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3. TEMPERATURE NICHE SHIFT OBSERVED IN A LEPIDOPTERA POPULATION UNDER ALLOCHRONIC DIVERGENCE.

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3. H. Santos, M. R. Paiva, C. Tavares, C. Kerdelhué, M. Branco (2011) Temperature niche shift observed in a Lepidoptera population under allochronic divergence. *Journal of Evolutionary Biology*, 24, 1897–1905. doi: 10.1111/j.1420-9101.2011.02318.x

Abstract

A process of adaptive divergence for tolerance to high temperatures was identified by using a rare model-system, consisting of two sympatric populations of a Lepidoptera (*Thaumetopoea pityocampa*) with different life-cycle timings, a "mutant" population with summer larval development, Leiria SP, and the founder natural population, having winter larval development, Leiria WP. A third, allopatric population (Bordeaux WP) was also studied. First and second instar larvae were experimentally exposed to daily-cycles of heat treatment reaching maximum values of 36, 38, 40 and 42ºC; control groups placed at 25ºC. A lethal temperature effect was only significant at 42ºC, for Leiria SP, whereas all temperatures tested had a significant negative effect upon Leiria WP, thus indicating an upper threshold of survival c.a. 6ºC above that of the WP. Cox regression model, for pooled heat treatments, predicted mortality hazard to increase for Leiria WP (+108%) and Bordeaux WP (+78%) in contrast with Leiria SP; to increase by 24% for each additional ºC; and to decrease by 53% from first to second instar larvae. High variability among individuals was observed, a population characteristic that may favour selection and consequent adaptation. Present findings provide an example of ecological differentiation, following a process of allochronic divergence. Results further contribute to a better understanding of the implications of climate change for ecological genetics.

Key words: Adaptation, climate, insects, niche shift, sympatric speciation

1. Introduction

Speciation processes take a central role in ecology but still remain insufficiently studied (e.g. Nosil *et al.*, 2009). Sympatric speciation in particular, i.e., divergence without geographical isolation, is poorly understood although the topic has been attracting increased attention (e.g. Dieckmann & Doebeli 1999; Simon *et al*., 2000; Friesen *et al*., 2007; Knudsen *et al*. 2010). Furthermore, although some studies address sympatric speciation resulting from a diet switch or habitat specialization (e.g. Knudsen *et al*. 2010), allochronic differentiation, a particular case in which asynchronous reproductive periods lead to speciation, while the evolved populations remain and coexist in the same area (Alexander & Bigelow 1960), has scarcely ever been observed.

Allochronic speciation may be due to a temporal shift between populations reproducing in different years (e.g. Simon *et al*. 2000; Cooley *et al*. 2001; Ritchie 2001), in different seasons (Friesen *et al*. 2007; Santos *et al.* 2007: Yamamoto & Sota, 2009), or even at different times of the day (Miyatake *et al*. 2002). However, studies mostly describe the mechanisms of reproductive isolation through time, failing to clarify the resulting ecological and evolutionary consequences, at organism or population level. Yet, as a result of a life cycle phenological shift, a significant alteration of the environmental parameters driving selection pressures, such as temperature, natural enemies or food quality, among others, may occur. This can induce ecological differentiation of sister populations, by acting on traits that were not primarily involved in genetic differentiation.

The pine processionary moth, *Thaumetopoea pityocampa,* is a monovoltine conifer defoliator, widespread in the Mediterranean region. Adults emerge in the summer, mate and lay eggs within two to four days. The gregarious larvae hatch after ca. one month and development progresses until late winter, with the larvae sheltering inside self-spawn silk nests. Pupation takes place in the soil, where the pupae undergo an obligatory diapause. *T. pityocampa* exhibits a variable phenology across its range of distribution, with late adult emergence (August - September) and early pupation (January - February) occurring in the warmest regions, while early adult emergence (June - July) coupled with late pupation (March-April), is observed in populations colonizing the northernmost, or high altitude areas (Démolin, 1969b). In 1997, a population of *T. pityocampa* having a shifted life cycle was discovered in Mata Nacional de

Leiria (National Forest Park of Leiria) (Paiva, pers. observ.). In this population, hereafter called SP (summer population), the larvae develop in the summer, pupate in September and the adults reproduce in April-May. It was observed colonizing the same stands and host trees as individuals having the normal phenology of the winter population, hereafter called WP.

Previous research conducted on the genetics of both populations, showed that Leiria SP probably originated from Leiria WP in recent historical times, and that a possible mutation induced, in some individuals, a sudden phenological shift (Santos *et al.*, 2007, 2011). The two sympatric populations share the same major haplotype for the mitochondrial cytochrome oxidase 1, and one allele of the nuclear ITS1 marker which is endemic in Leiria region and separated from other *T. pityocampa* populations of the Iberian Peninsula. This fact excludes the hypothesis of Leiria SP being a cryptic species (Santos *et al*., 2007) and suggests the occurrence of a fairly recent differentiation. Estimating the date of divergence between the two populations would yet require further molecular data. Gene flow is severely hampered between the two sympatric populations which are kept reproductively isolated by separate mating seasons (Santos *et al*., 2011).

Following the phenological shift, the "mutant" Leiria SP became suddenly exposed to new climatic conditions. In particular, larval development shifted from winter to summer, so that in Leiria, between June and September, SP larvae are subjected to daily temperatures on average 6.6º C higher than those of the WP (Leiria Meteorological Station, Portugal).

Forecasts of population trends, under different climate change scenarios (e.g. (Crozier, 2004; Hickling *et al*., 2006; Parmesan, 2006a) evidence that temperature affects the ecology and biology of animal populations. Most studies focused on the description of observed shifts in geographic distributions (e.g. Denlinger & Yocum 1998; Parmesan 2006; Hickling *et al.*, 2006). Niche conservatism is generally implicitly hypothesized, (Thuiller *et al*. 2005) by taking into account the species current ecological requirements, in particular the realized niches thresholds. Nevertheless, niche divergence in space and time has also been predicted (*e.g*. Pearman, *et al*., 2008).

Different mechanisms allow insects to overcome unfavourable temperatures, namely physiological and biochemical adaptations, such as up-regulating heat shock proteins (Denlinger & Yocum, 1998; Neven, 2000; Angilletta *et al*., 2002); behavioural changes, such as micro-habitat selection (Breuer *et al.* 1989; Heinrich, 1995; Denlinger & Yocum, 1998), or aggregation

(Kovac & Schmaranzer, 1996; Breuer, 1997; Ruf & Fiedler, 2000; Jones & Oldroyd, 2006); and phenological alterations, mainly through the regulation of diapause induction or termination (Pullin, 1996; Bale & Hayward, 2010). Such mechanisms are, at least in part, under genetic regulation and could therefore evolve and enable adaptation to new environmental conditions. Within this context, functional genetic diversity is of particular importance, especially when it gives rise to phenotypic polymorphism.

A rare system, consisting of two sympatric *T. pityocampa* populations, SP and WP, was used to investigate the possible evolution of larval survival under extreme warm temperatures, and to test the hypothesis that ecological requirements may evolve within a shorter than usually considered timeframe, such as tenths of generations. Experiments were complemented with data from another WP population, originating from France, where larval development starts in the summer. Larvae should thus be expected to tolerate higher temperatures than those of Leiria WP.

The specific objectives of this study were to establish if: (i) high temperature survival would differ between the two *T. pityocampa* Leiria populations, SP and WP, thus indicating a process of ecological niche divergence; (ii) tolerance to high temperatures would differ between two "normal" WP populations, sampled from distant regions at different latitudes; (iii) high temperature tolerance might be considered a polymorphic trait, enabling selection to act.

2. Material and Methods

2.1 Larval origin

T. pityocampa larvae were obtained from eggs originating from three populations: i) the unique Leiria SP (39º50´N; 8º57´W; < 50m elevation); ii) the sympatric Leiria WP and iii) a natural population form Bordeaux, France (hereafter, Bordeaux WP), located at a higher latitude (44º44´N; 0º46´W; < 50m elevation) than Leiria. The three populations were chosen due to their different phenologies, being hence exposed to different ecological conditions, particularly during the early larval stages. By contrast, they occur on the same hosts, namely maritime pine *Pinus pinaster* Ait., at a similar altitudinal range (30-50m) and identical distance from the Atlantic Ocean. In the field, larval development starts in June for Leiria SP, in August for Bordeaux WP and in October for Leiria WP. In consequence, the average maximum temperatures (± s.e.)

experienced by the young larval stages (first to third instars) are 25.2 ± 0.3ºC, 21.7 ± 0.7ºC and 23.7 ± 0.7ºC, respectively for Leiria SP, Leiria WP and Bordeaux WP (Leiria Meteorological Station, 2002-2009; Bordeaux Merignac Meteorological Station, 2002-2010).

In order to minimize larval mortality due to environmental factors other than temperature, such as diseases and parasitism, as well as to standardize acclimatization conditions, egg batches were kept in the laboratories of ISA (Lisbon) at room temperature (25 ± 2ºC), until the larvae hatched. In all experiments, first and second instar larvae only were used (coded L1 and L2), since these stages are considered to be the most susceptible to extreme warm temperatures. L1 designates the larval stage running from hatching to the first moult, and L2 refers to the following one, ending with the second moult. L2 larvae were obtained from L1 larvae kept at room temperature and fed on maritime pine until the first moult.

2.2 Rearing temperature conditions

The experimental design was based on the temperature record for the summer months in Leiria region, between 2002 and 2009, during which 13 periods of hot summer days with daily maximum temperatures exceeding 35.5ºC were observed, one of which lasted for three consecutive days (Fig. 1). Each treatment consisted of three complete 24 hours temperature cycles, 16L: 8D, mimicking three consecutive extremely hot days, to test the effects of extreme maximum temperatures (MT) on larval survival.

During the eight hours of scotophase, temperature was kept 10°C below the tested MT; it was then gradually increased, from the beginning of the photophase, to reach the desired MT within six hours. Temperature was kept constant for the following four hours, corresponding to the expected length of the warmest daily period in the field, and progressively decreased during the remaining six hours (Fig. S1, supporting information). Preliminary experiments were conducted by placing Leiria SP larvae for 4h at a constant temperature of 38°C, followed by 3h of larval survey. Surprisingly, only 11 out of 98 larvae died under these conditions, while 5% died in the control group, so that the MTs used for the present work were then set at 36°C, 38°C, 40°C or 42°C.

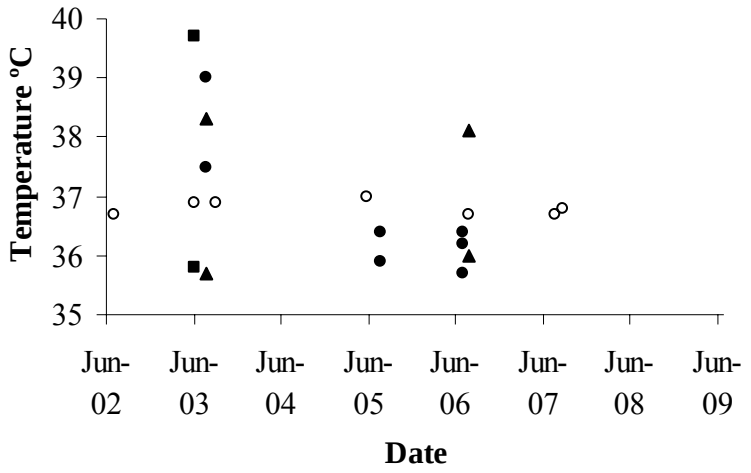

Figure 1 – Daily maximum temperatures above 35.5º C recorded in Leiria Meteorological Station, Portugal, from June to September, 2002 to 2009. Within the same year, two or more consecutive days of extreme temperatures are indicated with the same filled pattern; open circles represent single hot days.

Prior to the start of the first cycle, the larvae were acclimatized, for one hour to the initial temperature, i.e., 10°C below the respective MT. All cycles started at the beginning of the scotophase. Experiments were conducted in a climatic chamber FITOCLIMA, model 700 EDTU, with relative humidity fixed at 60%.

2.3 Experimental design

Larvae were kept inside acrylic boxes (18 x 12 x 12 cm), and fed daily with *P. pinaster* needles attached to a freshly cut branch, inserted into a wet floral foam Oasis®. Pine branches were previously sterilized by washing with a 0.53% bleach solution, rinsed and gently dried.

Each trial consisted of test boxes, exposed for three 24 hour cycles to one of the four MTs, as described above, and of the matching control boxes kept at room temperature in the laboratory (25±2ºC), for the same period. Room temperature was within the range of optimal temperature for larval development (Démolin, 1969a). Each replicate consisted of a test box and its corresponding control, comprising a sample of 20 larvae, collected at random from a mix of larvae hatched from five to seven egg batches (150 – 250 larvae usually hatch from each egg mass). In this way, treatment and control boxes were clearly associated and consisted of "sister"

groups of larvae of the same age and genetically closely related (although not from a single brood).

Four independent trials were conducted for each population and instar, according to the four MTs. On each trial the number of test boxes equalled the number of controls, which varied between trials, depending on the availability of larvae. Numbers for MT= 36ºC, 38ºC, 40ºC and 42ºC, were respectively: (10, 10, 14, 14) L1 and (7, 17, 12, 7) L2 for Leiria SP; (7, 22, 7, 7) L1 and (12, 7, 7, 8) L2 for Leiria WP; (7, 7, 7, 7) L1 and (7, 7, 7, 5) L2 for Bordeaux WP. Trials for Leiria SP and Leiria WP were repeated in 2008 and 2009, and then pooled. All other trials were conducted in 2009 only.

Dead larvae were counted daily and removed from the climatic chamber, fresh needles were added and the foams rehydrated. After three 24-hour cycles, the boxes were transferred to the laboratory to the same temperatures as during the pre-treatment period, and the larvae fed for an additional post-treatment period of one week. During this period and until the end of the trial, fresh pine needles were added and the foam rehydrated every 2nd day. Post-treatment survival was recorded every 2nd or 3rd day.

2.4 Data analysis

Kaplan-Meyer mean survival (± s.e.) were estimated for each experiment (Gehan 1975), and the respective survival curves plotted. Survival analyses were performed using Log Rank (Mantel-Cox) test statistics in which all larvae that were still alive at the end of the experiments were considered as censored data (in survival analyses, censored data are observations for which death is not recorded). For each trial, statistical tests were applied to compare survival curves of test boxes to their corresponding controls. Tests were also performed to compare the survival of instars at each temperature for each population (i.e., L1 *vs.* L2 at a given MT). A Cox regression analysis, which provides robust inference for the regression coefficients of a hazard function dependent on explanatory variables (Lin & Wei, 1989), was further used to model the effects of temperature, instar and population, pooling heat treatments (36ºC to 42ºC). The hazard function $h_i(t)$, that is the potential for a death to occur at a particular time, was modelled assuming the equation:

$h_i(t)=h_0(t)\exp(b_0 + b_1.\text{temperature} + b_2 + b_3)$

Where:

$h_0(t)$ is the baseline hazard at time t;

b_j is the value of the j^{th} regression coefficient, as follows: 1- temperature (°C); 2 - instar (L1 = 0; L2 = 1); 3 - population (population 1: Leiria WP = 1, population 2: Bordeaux WP = 1) reference category Leiria SP.

For each population and MT, the differences in the proportion of larvae surviving between treatment boxes and respective controls were further compared between populations at day 3, corresponding to the end of the heat treatment period, and at day 11, the end of the post-treatment period. To reach this aim, we performed two-way ANOVAs using two factors, namely "population" with three modalities (Leiria SP, Leiria WP and Bordeaux WP), and "temperature", with four modalities corresponding to the four MTs. A post-hoc LSD test was used to compare population pairs. Different ANOVAs were used for each instar at day 3 and day 11. Prior to the ANOVA analysis, the homogeneity of variances was tested by the Levene's test.

3. Results

3.1 Comparison of heat treatment effects within each population and larval instar

The effect of exposure to high temperatures was assessed by first comparing the survival curves for each experiment (for any given combination of population-instar-MT) with the respective controls. Results are summarized in Table 1.

Concerning Leiria SP, a significant negative temperature effect was observed for the highest MT only (42ºC). No significant effect was detected either at 38°C or at 40°C, for any of the instars. Unexpectedly, survival was significantly higher at MT = 36°C for L1 larvae, in comparison to the corresponding control (Table 1, Fig. 2A).

Concerning Leiria WP, L1 were negatively affected by all tested MTs, including 36ºC, while L2 had a significantly lower survival at MT = 40ºC and MT = 42ºC. No significant effect of MT=36°C or MT=38°C on L2 survival occurred. Finally, for Bordeaux WP, survival was significantly lower only for L1 at MT = 38ºC and MT = 42ºC, and for L2 at MT = 38°C, 40°C and 42°C.

Table 1. Results of the Log Rank (Mantel-Cox) tests comparing the survival curves of larvae at each tested MT to the corresponding controls, Wald qui-square (df=1) and p values, for each population and instar, 2008-2009.

MT	**36ºC**		**38ºC**		**40ºC**		**42ºC**	
Instar, population	Wald	p	Wald	p	Wald	p	Wald	p
First instar								
Leiria SP	6.59	***0.010***	0.55	0.459	1.41	0.236	114.70	**0.000**
Leiria WP	19.57	**0.000**	68.65	**0.000**	21.01	**0.000**	19.35	**0.000**
Bordeaux WP	0.00	0.964	3.92	**0.048**	2.96	0.085	60.40	**0.000**
Second instar								
Leiria SP	1.72	0.190	2.44	0.118	1.17	0.280	13.95	**0.000**
Leiria WP	3.44	0.063	1.68	0.194	4.21	**0.040**	67.04	**0.000**
Bordeaux WP	1.77	0.183	6.57	**0.010**	7.16	**0.007**	10.69	**0.001**

Bold regular: significant negative effect of the heat treatment; bold italic: significant positive effect of the heat treatment.

High variability at individual level was encountered in all groups (Fig. 2A & 2B). In all experiments, a gradual decrease in survival was observed over the treatment and post-treatment periods, rather than a sudden increase in mortality rate. For MT = 42ºC, between 5 and 75% of the individuals survived until the end of the pos-treatment period, depending on the population and instar (Fig. 2B). However, L1 larvae from Leiria WP, exposed at MT=42°C, proved to be an exception since 100% mortality was observed after day 10 (Fig. 2B).

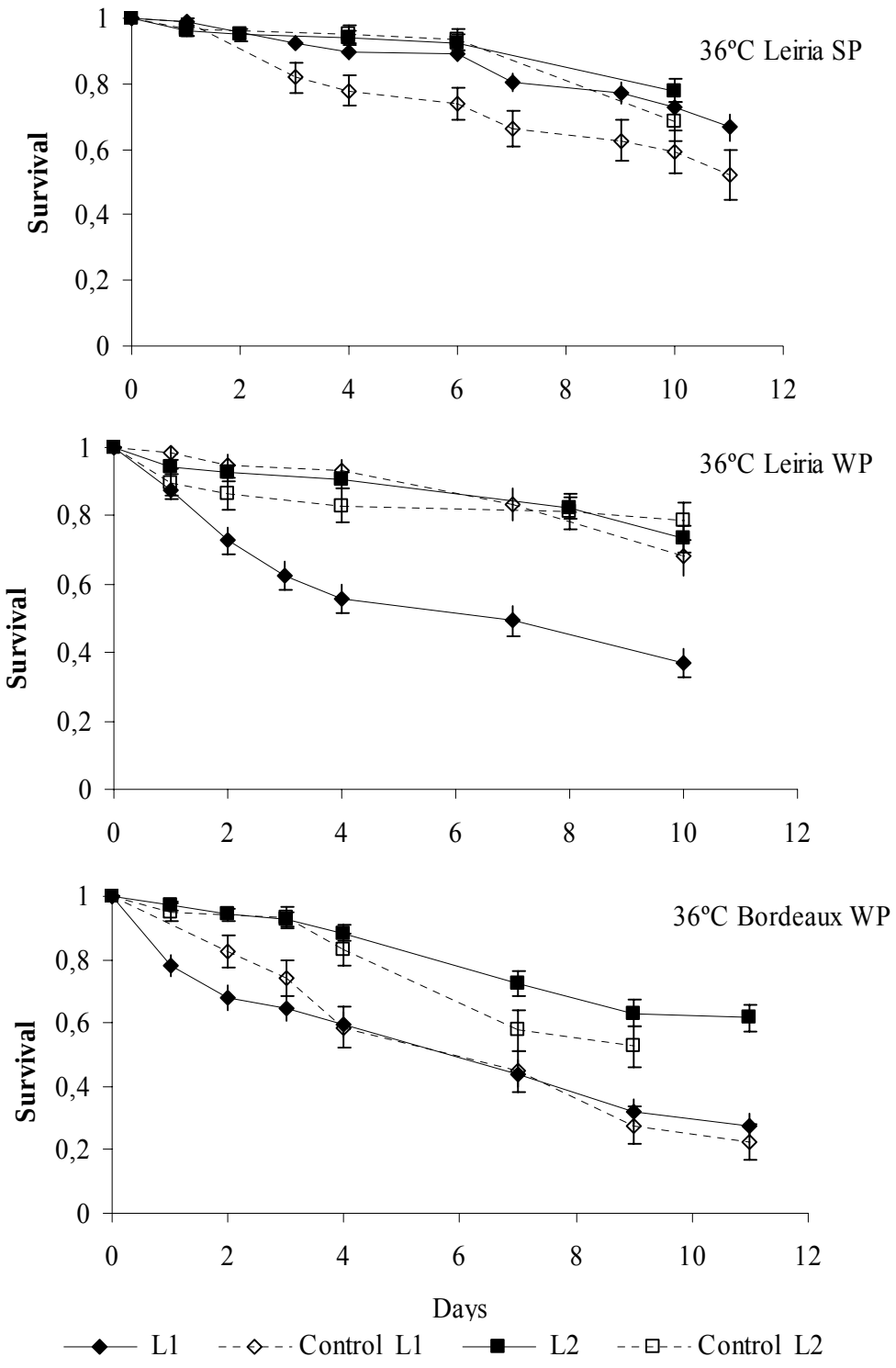
36ºC Leiria SP
36ºC Leiria WP
36ºC Bordeaux WP
Survival
Days
L1
Control L1
L2
Control L2

Fig. 2A

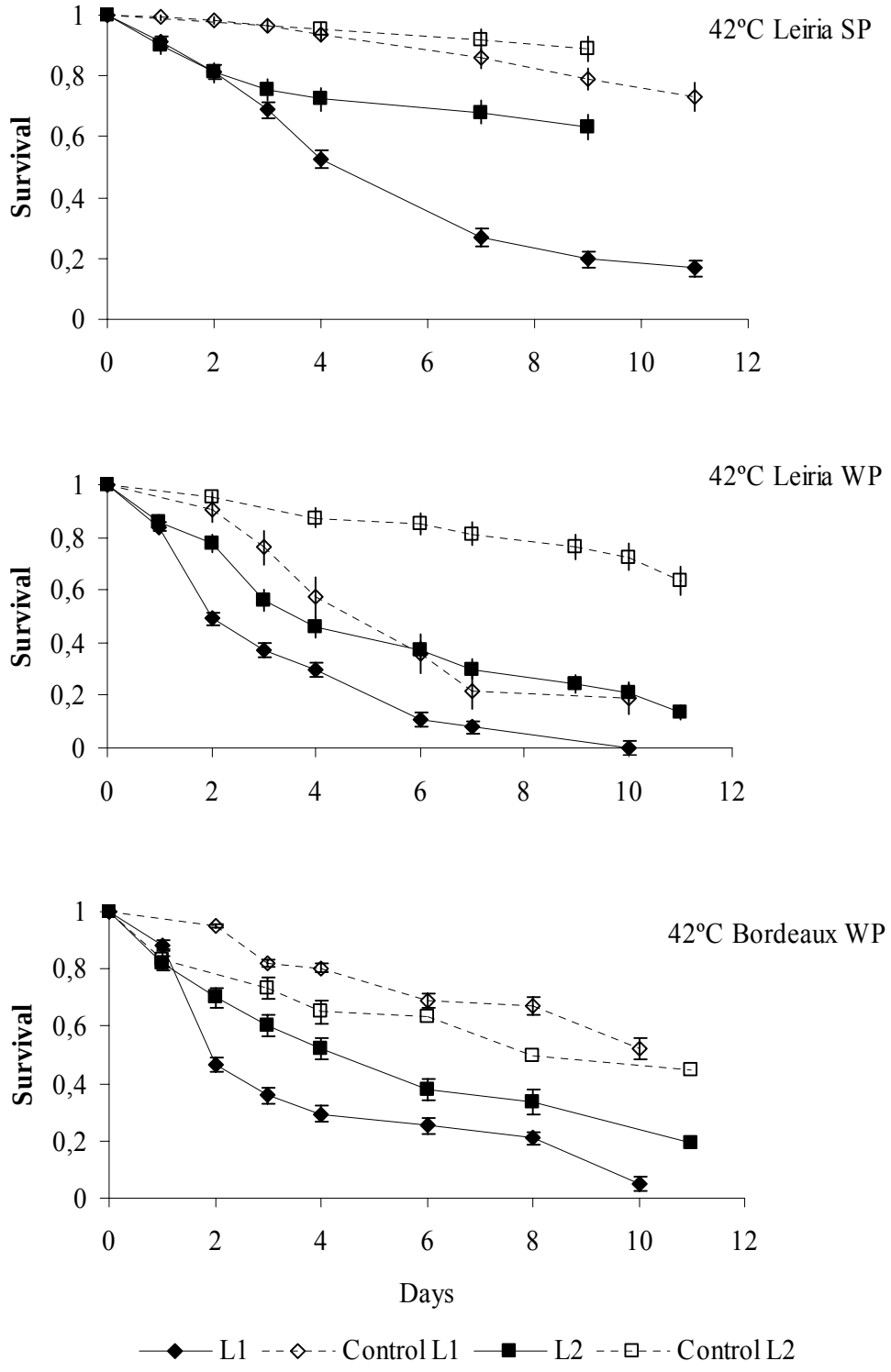

Fig. 2B

Fig 2 - Kaplan-Meyer survival estimates (± s.e.) for *T. pityocampa* L1 and L2 instar larvae, originating from Leiria SP (LSP), Leiria WP (LWP) and Bordeaux WP (BWP), subjected to temperature treatments: 2A: MT = 36º C and corresponding controls. 2B: MT = 42º C and corresponding controls.

3.2 Comparison of heat treatment effects between instars and among populations

Results of the statistical analysis performed showed that L1 were in most cases more susceptible to high temperatures than L2, having a significantly lower survival at a given MT. Yet, for Leiria SP, both instars survived similarly at MT = 36ºC and MT = 38ºC (Table 2).

Table 2. Results of the Log-Rank tests comparing survival rates of L1 and L2 *T. pityocampa* larvae for each maximum temperature (MT) and population tested, Wald qui-square (df=1) and p values.

MT	Population	Wald	p
36ºC	Leiria SP	2.92	0.087
	Leiria WP	41.77	**0.000**
	Bordeaux WP	39.30	**0.000**
38ºC	Leiria SP	0.26	0.609
	Leiria WP	72.81	**0.000**
	Bordeaux WP	17.09	**0.000**
40ºC	Leiria SP	10.77	**0.001**
	Leiria WP	15.21	**0.000**
	Bordeaux WP	14.75	**0.000**
42ºC	Leiria SP	53.63	**0.000**
	Leiria WP	32.61	**0.000**
	Bordeaux WP	23.10	**0.000**

Bold regular: Survival rate of L1 is significantly lower than survival rate of L2.

Survival decrease (i.e., the difference in survival rate between treatment and control) was significantly higher in Leiria WP than in Leiria SP, for L1 at day 3 ($F_{2,112}$ = 13.88; p <0.001) and for L1 and L2 at day 11 ($F_{2,112}$ = 4.31, p = 0.016 for L1; $F_{2,93}$ = 3.36, p = 0.039 for L2) (Fig. 3). No significant difference was found for L2 at day 3 when comparing Leiria SP to Leiria WP ($F_{2,93}$ =1.29; p = 0.281). Concerning Bordeaux WP, the decrease in survival was ranked between Leiria WP and Leiria SP (Fig. 3). Since the interaction term temperature*population was in general not significant (L1 at day 3 - $F_{6,112}$ = 2.10; p = 0.059; L2 at day 3 - $F_{6,93}$ =1.82; p = 0.103; L2 at day 11 - $F_{6,93}$ = 1.36; p = 0.240), except for L1 at day 11 ($F_{6,112}$ = 9.44; p < 0.001), the

differences between populations for each MT, were tested for this particular case only. For all MTs, except MT = 42ºC, decrease in survival was higher for Leiria WP than Leiria SP, Bordeaux WP always ranked in the middle, as shown in Fig. 3 for pooled values. For Leiria WP MT=42ºC, an unexplained high mortality was also observed in the control group, so that results cannot be coherently interpreted.

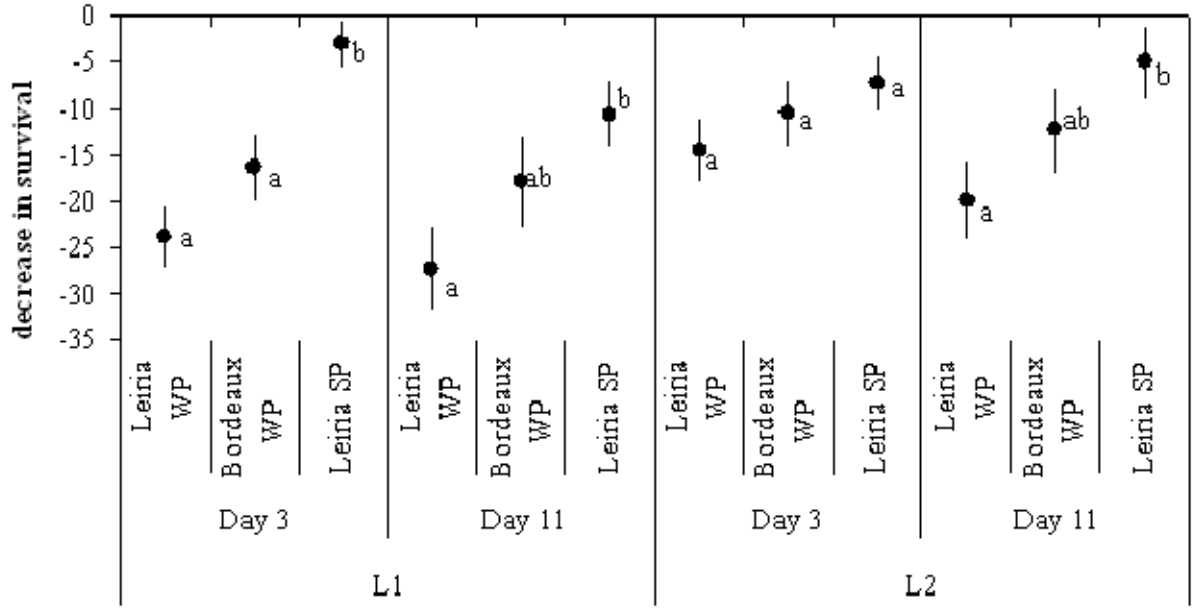

Fig. 3 - Comparison of mean decreased in survival (± s.e.), i.e. difference in observed survival between treatment and control for all tested MTs, at day three (end of temperature treatment) and day 11 (end of the post-treatment period) between Leiria SP (LSP), Leiria WP *T. pityocampa* L1 and L2 larvae.

The Cox regression model to predict mortality hazard in function of the covariables temperature (ranging from 36ºC to 42ºC), instar and population, revealed that all coefficients were significant (Table 3).

According to the model, the mortality hazard increased by 24% for each additional ºC, while from the first to the second larval instar it was reduced by 53%. The highest total increase in mortality is predicted for Leiria WP in comparison with Leiria SP (108%), whereas for Bordeaux WP this increase was 78% (Table 3).

Table 3. Cox regression results for the pooled heat treatments data (36ºC to 42ºC): Hazard function regression coefficients (B) and standard error (in brackets), and the respective mortality hazard increase.

Variable	B (s.e.)	Wald df=1	p	Exp(B)	Mortality hazard
Temperature (ºC)	0.22 (0.01)	382.3	0.000	1.243	+24%
Instar (L2 *vs.* L1)	-0.42 (0.04)	92.7	0.000	0.655	-53%
Leiria WP *vs*. Leiria SP	0.73 (0.05)	201.8	0.000	2.079	+108%
Bordeaux WP *vs*. Leiria SP	0.58 (0.06)	107.3	0.000	1.779	+78%

4. Discussion

The present study characterized larval survival at high temperatures for a "mutant" *T. pityocampa* population, recently founded by individuals originating from a typical winter population from Leiria (WP) (Santos *et al*. 2007). Due to an abrupt phenological change (Santos *et al*., 2007; 2011), the early larval instars of this summer population (SP) are now subjected to considerably higher temperatures than before. This unique situation allows for the study of rapid ecological divergence between two populations under allochronic differentiation. It is worth recalling that divergent natural selection, acting either by selection on a single trait, or on a larger number of traits, can be an important factor further promoting ecological speciation (Nosil *et al*., 2009). However, the occurrence of ecological divergence following genetic differentiation has hardly ever been documented (Savolainen *et al*., 2006).

Manipulative laboratory experiments with short life cycle species, such as fruit flies, bacteria and fungi, reared for several generations under contrasting environments, revealed that constraints can drive the rapid evolution of ecological traits (Maynard-Smith, 1956; Bennett & Lenski, 1999; Kassen, 2002). Further, a genetic basis for heat tolerance was inferred from laboratory selection trials with *Drosophila* species (Huey *et al*., 1991; Cavicchi *et al*., 1995; Hoffmann *et al*., 1997). However, regarding populations with longer life cycles the knowledge gap is substantial.

The evidence now presented comes from a natural situation, for a species with a longer life cycle. By comparatively studying survival at high temperatures in two *T. pityocampa* winter

populations, from regions located at different latitudes, Leiria and Bordeaux, which evolved under different selection pressures and have slightly different phenologies, it was concluded that tolerance to high temperatures is a rapidly evolving selected trait, showing high variability at individual level.

4.1 Population divergence for temperature tolerance thresholds: adaptation and possible rapid evolution

T. pityocampa larval survival differed among three populations tested, regarding tolerance to high temperatures. Tolerance was highest for Leiria SP and lowest for Leiria WP. Results are in accordance with the range of temperatures recorded locally, at the time of larval development, for each population and region: Leira SP, July - September, average maximum temperature (a.m.t.) 25.2ºC; Bordeaux WP, August – October, a.m.t. 23.7ºC; Leiria WP, September November, a.m.t. 21.8ºC. L1 larvae from Leiria SP are further subjected to maximum temperatures exceeding 35.0ºC, a temperature to which no other population from *T. pityocampa*, or from its closely related T. *wilkinsoni* is normally exposed. In Mediterranean areas with very hot summers, such as Northern Africa, or the Middle East, oviposition occurs in October and consequently larval development takes place under cooler conditions. At these lower latitudes, pupae spend the summer in the soil, where temperatures are lower, this stage also being less susceptible than all the others to extreme temperatures (Pullin, 1996).

A survival rate of over 60% was observed at 36º C for L1 larvae of the three populations, in contrast with data for south-eastern France, where L1 larvae experienced 100% mortality when daily temperatures reached 32°C (Démolin, 1969a). Here again differences in temperature tolerance could be due to local adaptation, as larval development in south-eastern France starts under moderate temperatures, in the autumn. Survival at 36°C was even higher than at control temperature, an unexpected result that could be due to a sterilization effect by the high temperature, upon pathogens that normally infect the larvae (Blanford & Thomas 1999).

In this study, *T. pityocampa* individuals were sampled from and reared on *P. pinaster*, the most frequent host in Portugal and south-western France. The ecological differences observed are thus not due to a host effect, and can be interpreted as local adaptation to climate by the early larval stages. Moreover, results show that variability exists at individual level, as mortality

occurred throughout the experiments with some individuals dying very early after exposure to high temperatures, whereas others survived at least for one week after the end of the experiment. Present results suggest that thermal tolerance was quickly acquired by a cohort of *T. pityocampa* SP founding individuals, from which enough survived to secure population viability. The mechanisms underlying these observations still need to be investigated. However, within population variability in tolerance to high temperatures is a trait that could rapidly evolve under strong evolutionary constraints (e.g. Thuiller *et al*. 2005). It can be hypothesized that physiological adaptations should be involved, such as divergences in the genetic mechanisms up regulation heat-shock and stress-related genes, as observed in other insects (Mahadav *et al*., 2009). To determine the occurrence of possible pleiotropy between phenology and thermal tolerance would necessitate further quantitative genetics, or genomic studies.

Phylogeographic studies have shown that *T. pityocampa* and *T. wilkinsoni*, are genetically highly structured in space at different spatial scales (Salvato *et al*., 2002; Simonato *et al*., 2007; Kerdelhué *et al*., 2009b). Results demonstrated the existence of local adaptation to temperature, suggesting that it probably occurs within the whole range of the species complex, and that this trait is variable for different populations and different regions where local climatic conditions differ. Phenotypic characteristics observed in one population should thus not be extrapolated to other populations. The same may apply to other traits, such as cold tolerance.

4.2 Implications for modeling population expansion

Summer temperatures in Leiria region are, on average, 4ºC lower than in neighbouring areas located 30 km inland, a fact which may have favoured the maintenance and development of the "mutant" Leiria SP, and could explain that it remains confined to a quite narrow geographical range. The highest maximum temperature observed in Leiria, between 2002 and 2009 was 39.7ºC, in one day only (Fig. 1). Nevertheless, larval survival of Leiria SP was not affected by temperatures up to 40°C, and about 20% and 70%, respectively of the L1 and L2 individuals, still survived for three consecutive days at a maximum temperature of 42ºC. This suggests that adaptation to hotter summers can be expected and that temperature constraints might not preclude SP expansion beyond coastal areas, into more continental or Mediterranean regions.

Models forecasting the dynamics of insect species under changing scenarios generally use parameters extrapolated from one, or a few locations only. Models usually under-estimate, or

even ignore geographic variations of adaptive traits and thus of the realized niche breadth among populations (Sutherst & Maywald 1985; Baker *et al*. 2000), which can be relatively large, especially near range boundaries (Oliver *et al*. 2009). From an applied perspective, present results draw attention to the fragility of forecasts assuming spatial and/or temporal niche conservatism (e.g. Thuiller *et al*. 2005), without considering evolutionary processes.

In conclusion, this study evidences that rapid adaptation to higher temperatures is most likely to occur in insect species. In a field mutant population, an increase in 6ºC of the upper temperature threshold was observed to have evolved in a relatively short period of time. Results can find application within the context of climate change scenarios, suggesting that some populations may remain *in situ*, even when ecological parameters attain values surpassing the species thresholds of tolerance.

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SUPPORTING INFORMATION:

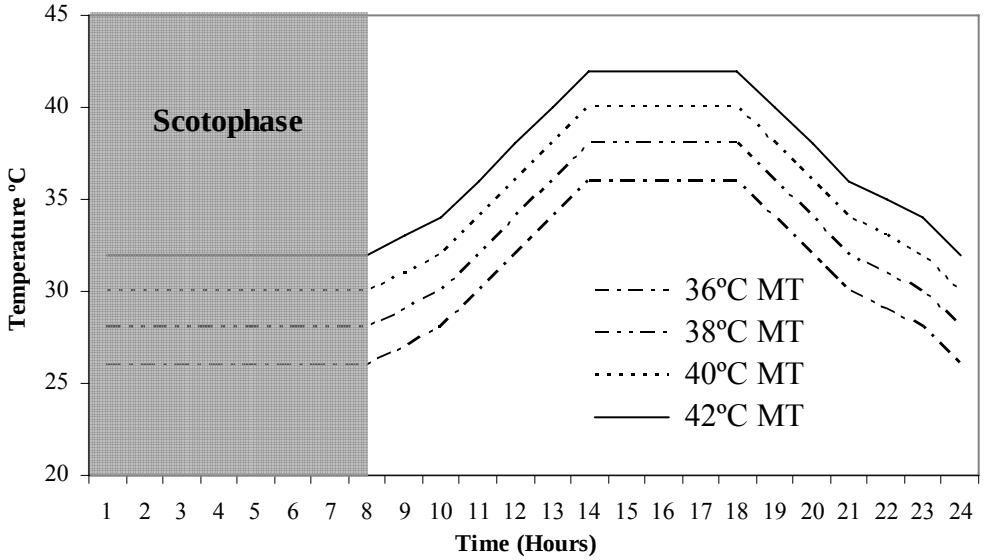

Figure S1. Climatic chamber 24 hour-temperature cycles. For each maximum temperature MT, trials correspond to three consecutive 24-h cycles.

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4. ECOLOGICAL DIVERGENCE IN THE EGG STAGE IN A LEPIDOPTERA POPULATION UNDER ALLOCHRONIC DIFFERENTIATION

Ecological Entomology
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4. H. Santos; M.R. Paiva; S. Rocha; C. Kerdelhué; M. Branco (Accepted) Ecological divergence in the egg stage in a Lepidoptera population under allochronic differentiation. ***Ecological Entomology.***

Abstract

1. Theory of ecological speciation predicts that divergent natural selection will reinforce speciation in populations subjected to different environmental pressures. The traits affected may include morphological, physiological and ecological ones.

2. A population of the pine processionary moth *Thaumetopoea pityocampa* ongoing allochronic speciation is here analyzed. The shifted population reproduces in the spring and develops in the summer, whereas the populations with typical phenology reproduce in the summer and have winter larval development. The two sympatric populations are thus reproductively isolated through time, although they share the same space and host species.

3. In this study we analyze divergences in the egg stage between the two sympatric populations, as well as by comparison with seven other allopatric winter populations, namely: female fecundity, egg size, egg covering, egg parasitism.

4. Females of the summer population laid a smaller number of larger eggs. The scales covering the egg batches differ significantly in shape and colour. A release from specific egg parasitoids was observed in the shifted population: parasitism rates are lower and generalist parasitoids represent over 95 % of the total parasitism.

5. Results evidence the occurrence of rapid differentiation of the phenotypic characteristics and life history traits in the egg stage of the shifted population. Data further support the evolutionary hypothesis that divergence of such characteristics is speeded up and strengthened by exposure to different ecological constraints. This work contributes to an insight on the rarely documented process of sympatric speciation, regarding how ecological divergence may follow the process of allochronic reproductive isolation.

Keywords: sympatric speciation; morphological traits; egg parasitoids; egg size; scale covering; fecundity; *Thaumetopoea pityocampa*; evolution

1. Introduction

Understanding the genetic, phenotypic and ecological differentiation occurring during speciation processes is of major relevance in evolutionary biology. Two main types of processes are predicted to drive speciation, mutational, when genetic mutations and genetic drift dictate chance occurrence and fixation of different alleles, or ecological, by adaptation to different environments or niches (Schluter, 2001; Schluter, 2009). Nonetheless, even when genetic mutations occur, subsequent ecological divergence due to different environmental pressures is important to complete speciation (Coyne & Orr, 1998; Turelli *et al.*, 2001; Nosil *et al.*, 2009; Schluter, 2009; Matsubayashi *et al.*, 2010). According to the theory of ecological speciation, factors such as food availability, shelter, intra-specific competition, climate and predators can be determinant selection pressures in the processes through which populations diverge and eventually become new species (Ridley, 2004).

It is generally accepted that reproductive isolation is usually the first cause leading to genetic population divergence. On the other hand, populations that are either separated by geographical or ecological barriers will evolve under different selection pressures, and speciation can then occur as a by-product of ecological adaptation (Schluter *et al.*, 2001; Rolán-Alvarez, 2007).

Regardless of the mechanism causing two populations to split, once they become subjected to different selection pressures, ecological differentiation will reinforce the speciation process, mainly through specialization and counter-selection of hybrids (Rundle & Nosil, 2005). Allochronic speciation is a particular case of sympatric speciation caused by asynchronous reproductive periods. In allochronic speciation, reproductive isolation occurs because individuals of different groups mate at different times of the year, season, or even of the day, and consequently become reproductively isolated. Consequently, the different life stages, either during reproduction or post-mating development, may also develop under different environmental conditions, particularly if they occur in different seasons. Ecological divergence may thus reinforce the process of allochronic speciation, although at population level, the consequences remain essentially unknown. This type of speciation has been mainly documented

in invertebrates (Marshall & Cooley, 2000a; Cooley et al., 2001a; Abbot & Withgott, 2004a; Yamamoto & Sota, 2009a; Ording et al., 2010; Santos et al., 2011a); yet one case of a seabird is also known (Friesen et al., 2007b).

The winter pine processionary moth *Thaumetopoea pityocampa* Den. & Schiff. (Lepidoptera, Notodontidae) is a univoltine species. Adults emerge over a period of 30 to 45 days during the summer months, each adult living only for three to four days. Reproduction and egg laying thus occur rapidly after emergence, and the embryonic development lasts about three weeks. The species is gregarious in the larval stage that normally develops during the autumn and winter, the larvae spinning silk nests in the tree crowns. By the end of the winter the larvae migrate to pupate in the soil, undergoing an obligate diapause, until the following summer. Most of the populations present this typical biological cycle and are hereafter called winter populations (or WP), as a reference to the time of the conspicuous larval development. By contrast, a unique population, hereafter called summer population (or SP), was discovered in an outbreak situation in Leiria, Portugal, in 1997 (Paiva, personal observation) and has been observed at high densities ever since. A typical WP also occurs in this forest. In the peculiar SP, larvae develop during the summer rather than winter, thus strongly differing from the typical life cycle (Pimentel et al., 2006b). Adult emergence and reproduction of the SP occur in the spring, between April and June. After completion of the larval development in the summer, pupation occurs in September. In consequence, an interval of 15 – 21 days occurs between the end of the reproductive period of the SP and the onset of that of the Leiria WP, hence causing reproductive isolation by time (Santos et al., 2007b). Mitochondrial cytochrome oxidase 1 and the nuclear ITS1 marker have shown that the two sympatric populations have recently differentiated (Santos et al., 2007b; Santos et al., 2011a). Further, microsatellite markers suggested that Leiria SP was founded by individuals from Leiria WP that experienced a phenological shift, and that the level of gene flow is now severely reduced between the two sympatric populations (Santos et al., 2007; Santos et al., 2011a).

Due to a divergent phenology, this new population is subsequently facing environmental and ecological pressures that differ from those experienced by the sympatric (and plausibly ancestral) WP. In particular, each life stage is now exposed to different climatic conditions and pressure from natural enemies. In a previous study performed with the same model population, we showed a local adaptation in the larval stage of the SP that develops under much higher

temperatures. Consistently, the young larvae of the shifted SP were more tolerant to high temperatures, surviving at up to 40ºC, whereas survival of the typical WP was hampered at 36ºC (Santos *et al.*, 2011b). In the present paper, we aimed to assess ecological divergence between SP and WP during the early post-mating period, focusing on egg and egg batch characteristics. We targeted several morphological traits that may affect embryonic survival, as well as the communities of egg parasitoids. We hypothesize that egg exposure to different climatic conditions and a probable release from specific natural enemies due to the phenological shift may reinforce the ecological divergence of the SP, both from the sympatric WP as well as from other WPs.

Several parasitoid species are known to attack the eggs of *T. pityocampa*, mainly *Baryscapus servadeii* (Dom.) (Hymenoptera, Eulophidae) a specific parasitoid and the generalist *Ooencyrtus pityocampae* (Mercet) (Hymenoptera, Encyrtidae) and *Trichogramma embryophagum* (Hartig) (Hymenoptera, Trichogrammatidae) (Biliotti, 1958; Tiberi, 1990; Tsankov *et al.*, 1996; Schmidt *et al.*, 1997; Schmidt *et al.*, 1999; Tsankov *et al.*, 1999; Mirchev *et al.*, 2004). Since the SP reproduces in a season different from the WP, an asynchrony with the activity of the specific egg parasitoids may occur. According to this hypothesis, egg parasitism should be low in the SP, which consequently may affect the egg traits through a release of the selective pressures from natural enemies. We further hypothesize that generalist, rather than specialist parasitoids, may have a higher relative importance on egg parasitism of the SP.

The females of *T. pityocampa* lay their eggs around pine needles, in a single egg batch, wrapped and protected by the female's abdominal scales (Schmidt *et al.*, 1999). This covering creates a microhabitat that enhances egg hatching success due to its effect on temperature (Milani, 1990). Covering may further disguise the eggs, or pose a physical barrier protecting against parasitism (Schmidt *et al.*, 1997; Pérez-Contreras & Soler, 2004). As embryonic development occurs under colder temperatures for the SP than for the WP (May vs. August), we expect that the main selective pressures on the egg covering behaviour in the SP will favour traits that improve temperature conditions for the embryos. On the contrary, due to the phenological shift and consequent "enemy-free space" in which the SP develops, we expect the evolution of the egg batch morphology to be less constrained by parasitoids than in the WP.

Both female fecundity and egg size directly affect fitness, but a trade-off is usually observed between these two traits (Fox & Czesak, 2000; Fischer *et al.*, 2003; Czesak, 2003 ; Pérez-Contreras & Soler, 2004), both of which might be influenced by climate and natural enemies. In typical WPs, the number of larvae per nest, which is proportional to female fecundity, is most important to guarantee overall nest survival during winter (Pérez-Contreras *et al.*, 2003). However, since SP larvae develop under warmer temperatures (Pimentel et al., 2006b), nest size and thus female fecundity might be less decisive for the fitness of the SP. On the other hand, egg size determines the amount of reserves that the embryo will have to feed and grow and consequently the size of neonate larvae. According to the ecological theory, selection may favour larger eggs under colder environments (Fischer *et al.*, 2003) and thus an adaptive response of egg size to temperature could be expected in the SP. Moreover, this trait might be most relevant in the case studied, since bigger larvae survive better when feeding on *Pinus pinaster* (Zovi *et al.*, 2008), which is the dominant pine species in Leiria. Since larvae are released from the cold winter pressure, the summer population should evolve towards the production of fewer and larger eggs, a trade-off frequently observed in other insects (Atkinson *et al.*, 2001; Fischer & Fiedler, 2001; Fischer *et al.*, 2003).

Considering the above traits and corresponding hypotheses, we thus compared the phenotypic traits related to the egg stage between the SP and several WPs, regarding: i) female fecundity; ii) egg size; iii) egg batch covering (scale dimensions, shape and colour); iv) parasitism rates and v) composition of the parasitoid communities. Experiments aimed at demonstrating that this unique population, which has a divergent reproductive time, presently undergoes divergent evolutionary processes shaped by different ecological pressures. Such findings would provide support for the hypothesis of ecological divergence and possible reinforcement, following allochronic reproductive differentiation.

2. Material and methods

2.1. Female fecundity and egg size

Egg batches from eight *T. pityocampa* winter populations (WPs) distributed along a latitudinal gradient in Portugal, as well as from the Leiria summer population (SP), were collected between 2000 and 2011 (Fig. 1 and Table 1). In total, 759 egg batches were collected. The number of eggs per egg batch was counted in the laboratory under a binocular microscope, for all egg batches.

Egg size was also compared between three populations, namely Leiria SP, the sympatric Leiria WP, and a geographically close WP from Apostiça chosen for being located in the same geographical region, about 150 km south from Leiria, at the same elevation and longitude. Further, this population presents a similar phenology to Leiria WP, and genetic differentiation between them is very limited (Santos *et al.*, 2011a). Egg size was calculated for 10 egg batches per population. For each egg batch, ten eggs were measured, using 1-2 eggs per line along the length of the egg batch. A binocular microscope and an ocular micrometer were used, following the procedure described in Zovi *et al*. (2008).

Figure 1- Map of Portugal with the location of the studied populations

Table 1. Populations of *T. pityocampa* studied: location, years and number of egg batches sampled.

Population	Coordinates (lat, long)	Elevation (m)	Years of sampling	N. of egg batches
Alcácer do Sal	38º 23'N; 08º 31'W	50	2002	12
Apostiça	38º 30'N; 09º 11'W	35	2000- 2005	163
Rio Frio	38º40N; 08º52W	20	2006	23
Azambuja	39º05N; 08º53W	100	2006	23
Barrada	39º25N; 08º03W	150	2007	13
Abrantes	39º 33'N; 08º 14'W	160-230	2000-2003	176
Leiria SP	39º 50'N; 08º 55'W	30-50	2000-2011	283
Leiria WP	39º 50'N; 08º 55'W	30-50	2000- 2005	36
Vila Real	41º 19'N; 07º 44'W	480	2003	30

2.2. Egg parasitism

Egg parasitism was determined for the nine *T. pityocampa* populations studied. To assess egg parasitism, egg batches were maintained at laboratory conditions, individually kept inside vials until emergence of the parasitoids ceased. All parasitoids that emerged were collected, identified and counted. The eggs were then uncovered by removing the scales and all hatched, unhatched, and parasitized eggs counted. As some parasitoids could have emerged in the field before egg batch collection, the number of parasitized eggs was determined by counting the number of eggs with parasitoids' emergency holes. This data was used for parasitism analyses, whilst the number and species of parasitoids that emerged were used to assess and analyse species frequencies.

2.3. Egg parasitoids and T. pityocampa phenology

According to Battisti (1989) and Schmidt *et al.* (1999), *T. pityocampa* egg parasitoids *B. servadeii* and *O. pityocampae* may have a generation without diapause, with adults emerging about two to three weeks after oviposition, allowing for a second oviposition while the host eggs

are still available, thus originating a generation which undergoes a diapause and emerges in the following year, coinciding with the egg laying season of the pine processionary moth. To determine whether the period of parasitoid emergence occurs when eggs of *T. pityocampa* are available, we monitored and characterized the male flight period in the field (that was used as a surrogate for egg laying) and followed the emergence of the diapausing parasitoids from the egg batches collected in the field.

Comparisons were made between the two closely related winter populations, Apostiça and Leiria WP, as well as with Leiria SP. Adult males were sampled in the field throughout the flight season, using funnel traps baited with synthetic pheromone dispensers (pityolure 40 mg), as described in Santos *et al*. (2007b).

2.4. Scales

Scale removal from the egg batches proved to be a difficult task since most scales were broken in the process. Scales were thus collected directly from the tip of the abdomen of virgin females that emerged in the laboratory, obtained from mature larvae collected in the field, from the populations of Apostiça, Leiria SP and Leiria WP. Twenty females of each population were used, each female supplying eight to twelve scales that were mounted between two microscope cover slips and fixed with tape. The preparations were then scanned and WinSEEDLE^{TM} 2008 software (Regent Instruments Inc., Canada) was used for analysing the images. Four biometric variables that are given by this software were selected: projected area (P_{area} - in mm^2); projected form coefficient ($Pform = 4\pi A/P^2$ where A = projected object area, P = projected object perimeter. It varies between 0 and 1, 1 being a perfect circle and 0 a filiform object (a line)); maximum straight width (W - in mm); and width to length ratio (W/L, L being the maximum straight length).

For colour analysis, 12 colour classes required by the software were defined by the authors from the universe of colours present in the scales sampled. Three groups of colours (light, medium and dark) were defined in each group, and four colour classes were chosen from the scales of each population, to represent up to the limit of 12 classes as much variety as possible within each group. The WinSEEDLE software calculates the percentage of the area occupied by

each class, assigning each pixel to one of the twelve classes defined. Orphan pixels (those that do not match exactly any of the defined classes) are automatically assigned to the closest class. Data resulting from this analysis were further aggregated into two mutually exclusive groups: dark colours and light colours. Results were expressed as percentage of scale area represented by light colours.

2.5. Data analysis

Data obtained from the egg batches were analysed using SPSS statistics version 17.0 and presented as mean ± standard error of the mean (SEM).

Female fecundity

The number of eggs per egg batch was analyzed by Generalized Linear Models (GLM), using a Poisson log linear link function, considering the population factor. A maximum likelihood estimation procedure was used. Differences between populations were analyzed by the Least Significant Differences (LSD) statistical test ($\alpha = 0.05$).

To relate the egg load with latitude and elevation, the geographic coordinates for each population were transformed into rectangular coordinates using the military Datum Lisboa system and correlated using Spearman's coefficient, ρ, with the mean values of each parameter for all populations. SP was eliminated from all analysis since the latitude and altitude is the same as for Leiria WP.

Egg size

A linear mixed model was used to analyze differences between populations regarding egg size. The eggs measured within the same egg batch were considered as repeated measurements, egg batches as subjects and populations as factors.

Parasitism

The number of eggs parasitized per egg batch was analyzed by Generalized Linear Models (GLM), using a Poisson log linear link function, considering as predictors: i) the factor

population; ii) the factor year (nested within population) and iii) the covariate number of eggs per batch. A maximum likelihood estimation procedure was used. The LSD test was used to compare the means between populations ($\alpha = 0.05$).
Parasitism was also related to latitude and elevation, using the same procedure as described for egg load.

To test for differences in the frequencies of the parasitoids, grouped as specialists and generalists, between the two reproductively isolated populations, a Pearson's chi-squared test was used.

Scales shape and colour

The five variables measured from the scales, four of which are biometric plus colour, were treated by a Principal Component Analysis (PCA) to differentiate groups. A discriminant analysis was then performed using the same five variables and predetermined groups (Leiria SP, Leiria WP and Apostiça). Two discriminant functions were estimated to separate the three groups. The probability of each individual being correctly assigned to its group was further determined.

3. Results

3.1. Female fecundity

Female fecundity varied between 16 and 322 eggs with significant differences between populations, Wald chi-square was 2335.55 (df = 8, $P < 0.001$). Leiria SP showed the lowest mean fecundity 139.4 ± 3.37 (Fig. 2).

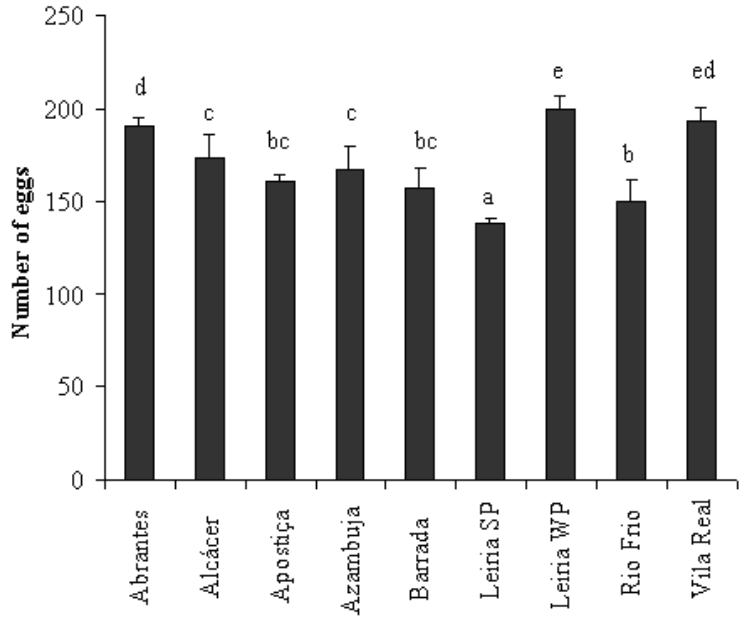

Figure 2 – Mean number of eggs per egg batch ± S.E. for eight *T. pityocampa* winter populations, including Leiria WP and the summer population, Leiria SP.

Pairwise comparisons showed that female fecundity of Leiria SP was significantly lower than all sampled winter populations ($P < 0.001$). Closest to Leiria SP was the population of Rio Frio, yet differing significantly by 11.42 ± 2.70 ($P < 0.001$) eggs per egg batch. By contrast, among the winter populations, the sympatric Leiria WP showed the highest mean number of eggs per batch (195.7 ± 8.90). Vila Real, located at the northern end of the latitudinal ranged studied, had the second highest fecundity, and the difference between Vila Real and Leiria WP was not significant ($P = 0.069$). All other WPs differed significantly from Leiria WP, indicating a lower fecundity (Fig. 2).

No significant correlation was found between the mean number of eggs per batch and either latitude ($\rho = 0.45$; $P = 0.26$), or elevation ($\rho = 0.31$; $P = 0.46$).

3.2. Egg size

Egg size was significantly higher for Leiria SP (1.22 ± 0.004 mm), in comparison with both Leiria WP (1.16 ± 0.004 mm) and Apostiça WP (1.07 ± 0.005 mm) ($F_{,2,273} = 314.25$; P

<0.001). The two winter populations also differed significantly (P <0.001; 95% CI of difference between the two Leiria populations was [4.975; 7.338]). The ratio egg size / egg load did not differ significantly among populations ($F_{1,2}$ =1.94; P = 0.163).

An indicative estimate of egg volume was calculated by considering that egg shape could roughly be considered spherical and using the parameter egg size as diameter. Volumes of 0.95 ± 0.010, 0.82 ± 0.009 and 0.65 ± 0.009 mm^3 were obtained respectively for the SP, Leiria WP and Apostiça WP, translating the difference in the amount of reserves available for the development of the embryos.

3.3 Parasitism

The number of parasitized eggs per egg batch was highly variable (Fig. 3). Significant differences between populations (Wald chi-square = 1618.45, df = 8, P < 0.001), and between years within each population (Wald Chi-Square = 374.95, df = 11, P < 0.001) were observed. Additionally, the number of parasitized eggs increased significantly with the number of eggs per egg batch (b=0.002±0.0003, Wald Chi-Square = 76.32, df = 1, P<0.001). Leiria SP showed the lowest estimated mean number of eggs parasitized (0.07±0.006), followed by Leiria WP (5.62±0.542). 95% CI for the difference between Leiria SP and Leiria WP was [-6.61; -4.49]. Alcácer population had the highest number of parasitized eggs per egg batch, estimated marginal mean 43.87 ±1.892 (Fig. 3).

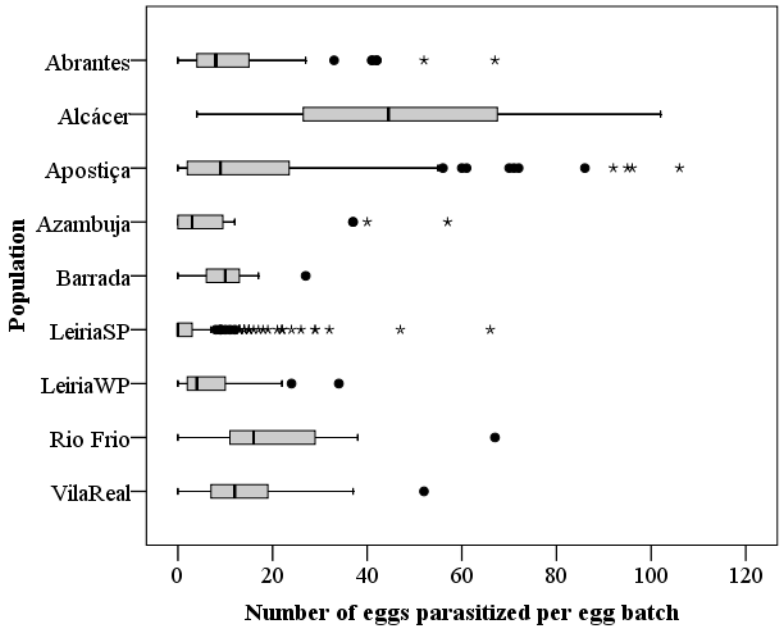

Figure 3 - Mean parasitism rates ± S.E for the egg batches of eight *T. pityocampa* winter populations, including Leiria WP and the summer population, Leiria SP.

A significant negative correlation was found between the mean percentage of parasitism and latitude (ρ = -0.71; P = 0.047), but correlations with elevation were not significant (ρ = -0.36; P = 0.38).

The main parasitoid species found in all samples, except for Leiria, were *O. pityocampae* and *B. servadeii*, although their relative proportions varied among populations. In Leiria, high proportions of *T. embryophagum* were observed both in the SP and in the WP, a species which was not found elsewhere (Fig. 4).

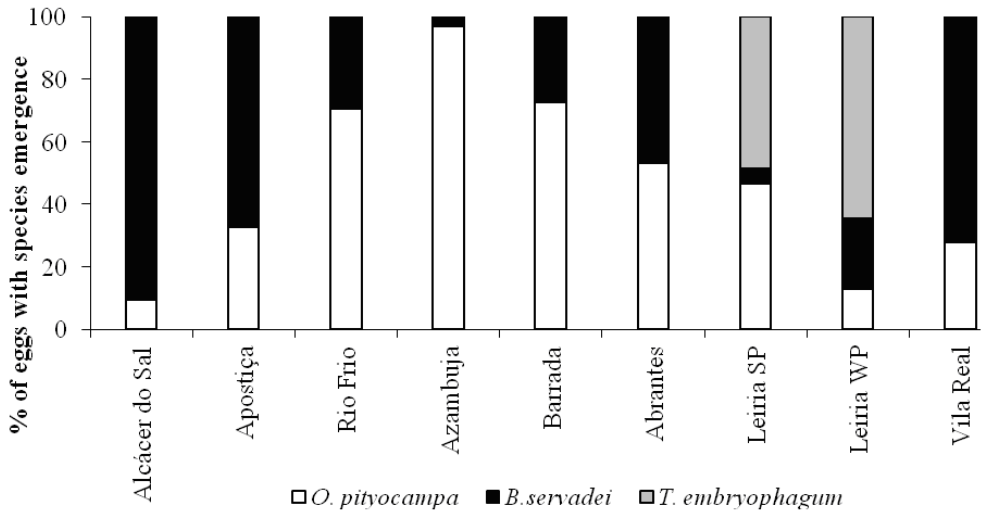

Figure 4 - Proportion of egg parasitoid species emerged for eight *T. pityocampa* winter populations, including Leiria WP and the summer population, Leiria SP.

O. pityocampae was also abundant in Leiria SP, representing 46.6% of the total parasitism, that is almost as high as for *T. embryophagum* (48.6%). The two Leiria populations differed significantly on the frequencies of the pooled generalists, *O. pityocampae* and *T. embryophagum*, and of the specialist *B. servadeii* (Chi-square = 76.87, df=1, $P < 0.001$). *O. pityocampae* emerged early in the season, having a main peak between March and June, synchronous with SP male flight and a second one in July – October, overlapping with the flight activity of the WP. *B. servadeii* was the second most important species in Leiria WP (20%), showing emergences well synchronized with the male flight period and marginally overlapping with the flight period of the SP (Fig. 5).

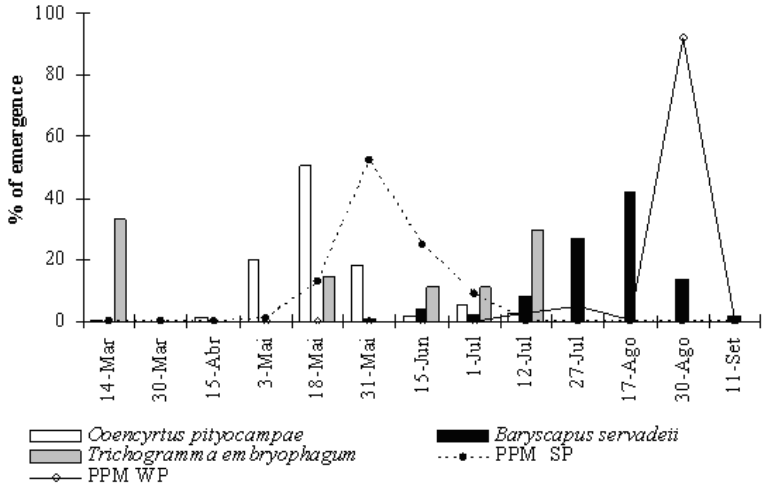

Figure 5 – Phenology of the egg parasitoid species observed for *T. pityocampa* Leiria summer population SP and adult flight periods of both Leiria SP and Leiria WP.

T. embryophagum emergence occurred over a wider period, as adults were observed from the end of the winter to the beginning of the summer.

3.4. Scales shape and colour

The scale variables analysed showed a consistent pattern of variation across the three populations studied. Thus, the values obtained for Leiria SP and Apostiça always ranked further apart while Leiria WP showed intermediate values. Furthermore, the phenotypic distances between Leiria WP and Leiria SP were always larger than between the two WPs (Table 2). The projected area and colour of the two winter populations did not differ significantly.

The multivariate PCA demonstrated a separation of Leiria SP from the two winter populations, Leiria WP and Apostiça, while the two winter populations overlapped (Fig. 6). A higher variability was observed in both Leiria SP and Leiria WP, than in Apostiça (Fig. 6).

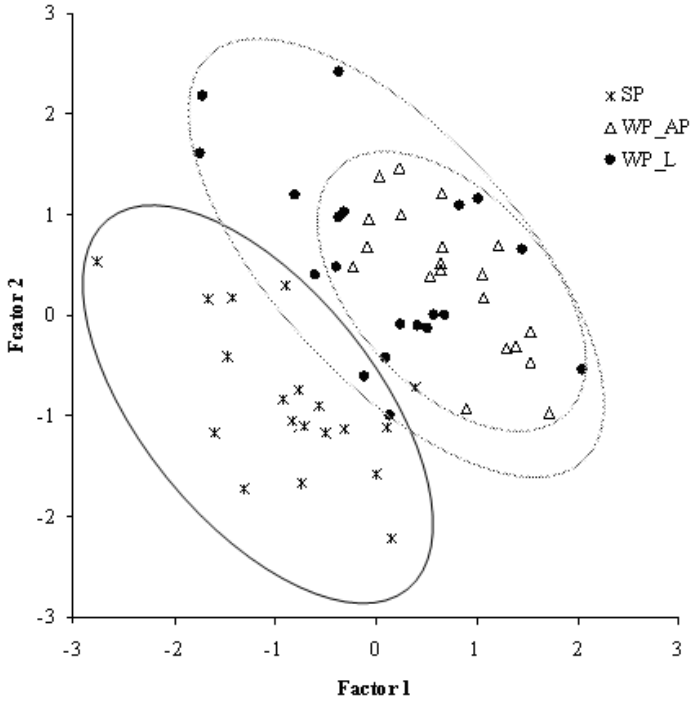

Figure 6 – Principal component analysis using four phenotypic variables of *T. pityocampa* egg scales: projected area; straight width, width /length ratio, projected form coefficient and colour, based on mean values (n = 10 x 20 females /population). The first and second PCA components explain 92% of the variance. Circles enclose populations for better visualisation of the separation.

Discriminant Functions could separate the three populations based on the scales' phenotypic measurements (Chi-square = 97.172, df = 10, $P < 0.001$ and 9.018, df = 4, $P = 0.061$, for first and second discriminant functions, respectively). Concerning Leiria SP, 95% of the observations were placed in the same group (one observation out of 20 was assigned to Leiria WP). For Leiria WP, 85% of the observations were correctly assigned (one was wrongly assigned to Leiria SP and two to Apostiça). Concerning Apostiça, 80% of the observations were correctly classified (four observations were assigned to Leiria WP).

On average, Leiria SP had smaller scales than the other two populations, with a more triangular shape, a coefficient form (0.60 ± 0.01) and length 2.5 higher than the width, while thinner and darker in colour (Table 2, Fig. 7).

Table 2. Pairwise comparisons using LSD (ANOVA) for the egg scales variables (Mean ± S.E., n = 10 x 20 females /population) of three *T. pityocampa* populations: different letters indicate significant differences at p< 0.05.

Variable	Area	Straight width	Width/Length ratio	Form coefficient	Colour
Leiria SP	3.04 ± 0.11 [a]	1.22 ± 0.04 [a]	0.40 ± 0.01 [a]	0.60 ± 0.01 [a]	44.51 ± 2.12 [a]
Leiria WP	3.69 ± 0.11 [b]	1.57 ± 0.04 [b]	0.52 ± 0.00 [b]	0.67 ± 0.01 [b]	67.10 ± 2.12 [b]
Apostiça	3.84 ± 0.11 [b]	1.69 ± 0.04 [c]	0.58 ± 0.01[c]	0.73 ± 0.01 [c]	67.04 ± 2.12 [b]
$F_{2, 57}$	15.93	48.99	86.74	50.81	37.91
p	<0.001	<0.001	<0.001	<0.001	<0.001
Phenotypic distance					
Leiria WP - Leiria SP	0.65	0.35	0.12	0.07	23.39
Apostiça - Leiria WP	0.15	0.12	0.06	0.06	0.06

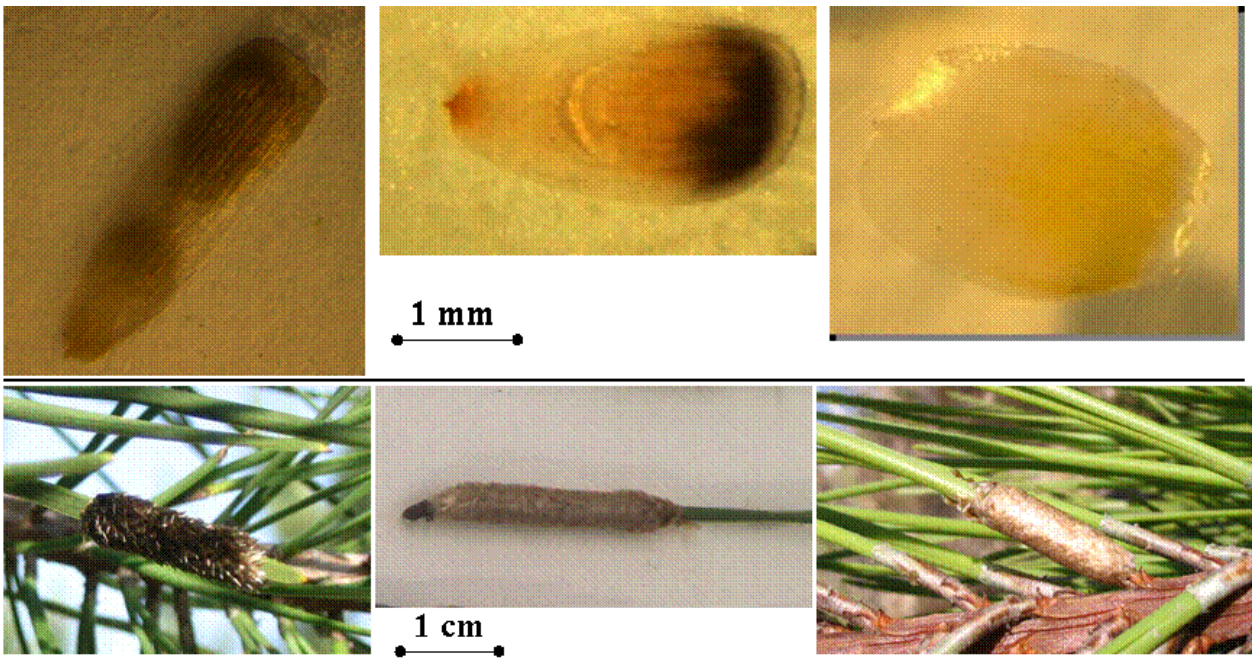

Figure 7 – Detailed images of the abdominal scales of *T. pityocampa* (above) and of the egg batches (below). Leiria summer population (left), Leiria winter population (center) and Apostiça winter population (right).

4. Discussion

Egg size and fecundity

In this work, a divergence of the egg traits between two populations of the pine processionary moth reproductively isolated by a different phenology was observed and studied. Independently of the mechanism causing the shifted phenology, since life stages develop in different seasons, they are subjected to different selective pressures, promoting their ecological differentiation. In particular, a lower fecundity, compensated by larger eggs, was observed in Leiria SP in comparison with all WPs.

A trade-off between egg size and fecundity is well established in the literature (e.g. (Fox & Czesak, 2000; Fischer & Fiedler, 2001; Gillooly *et al.*, 2002; Fischer *et al.*, 2003; Pérez-Contreras & Soler, 2004; Pimentel *et al.*, 2010). The latitudinal gradient described for the pine processionary moth in (Pimentel *et al.*, 2010) suggests that higher egg size occurs in the southern parts of the distribution range, contrary to what is usually observed for other Lepidoptera and insects in general. Early larval survival is favoured by increased egg/neonate larval size (Fox *et al.*, 1997; Fox & Czesak, 2000). Yet, near the northern range of distribution, winter harsh temperatures impose a high pressure, and due to the gregarious larval behaviour of *T. pityocampa*, the number of eggs in an egg batch will have a direct influence on colony size and winter nest survival (Pérez-Contreras *et al.*, 2003). This ecological constraint will favour a higher number of eggs in disadvantage of larger neonates in colder areas. Since SP larvae develop in the summer and face extreme hot temperatures, larval nests are not primarily used to resist low temperatures and thus the number of larvae per nest is probably not as critical as for the WPs. In agreement, a lower investment in silk production by the larvae is generally observed, and nests become looser structures (authors pers. obs.). By contrast, summer hot temperatures may act as a limiting factor for the survival of SP larvae, as evidenced in a previous study (Santos *et al.*, 2011b).

Selection may favour larger eggs under colder environments and thus a plastic response of egg size to temperature could be adaptive (Fischer *et al.*, 2003). The egg stage of Leiria SP is presently subjected to mean temperatures of 15.6 ºC by comparison with 19.7 ºC for the WP

when peak oviposition occurs, respectively in May and August (data 2001 - 2011, from www.snirh.pt). Therefore, it is plausible that the lower temperatures observed during the development of the embryo of Leiria SP may further favour larger eggs with increased chances of survival.

Moreover, SP larvae feed on a host with particularly tough needles, the maritime pine *P. pinaster* on which the size of the neonate larvae is a critical factor determining survival (Zovi *et al.*, 2008). This ecological factor might further reinforce the evolutionary trend towards fewer and larger eggs with higher reserves, a trait that might improve larval performance.

Egg covering

Egg covering by *T. pityocampa* females creates a microhabitat to protect the eggs from external environmental conditions and from natural enemies, especially parasitoids (Pérez-Contreras & Soler, 2004). A large variability within and among *T. pityocampa* populations was observed regarding the egg scales. In particular for Leiria SP, scales were smaller, thinner and had a more pronounced triangular shape than those of the populations with winter phenology. As the scales curl inwards a tight roof type adjustment is precluded, leaving some free space among them (Fig. 7) and increasing egg exposure to parasitoids. Although this trait might not be adaptive, no counter selection is acting, since egg parasitism is extremely low on this population. SP scales are also significantly darker than those of the two winter populations, which may contribute to raise egg temperature. Further research is needed to test this hypothesis.

Egg parasitism

Three egg parasitoid species were found in both Leiria populations. Yet, in contrast with the WP, a very high proportion of parasitism by generalist species was recorded on the SP by *T. embryophagum* and *O. pityocampae* while only a minor effect was caused by the specific parasitoid *B. servadeii* (4.9%). Specific parasitoids must closely adapt their life cycles and phenology to those of their host (Godfray *et al.*, 1994; Hawkins *et al.*, 1997; Van Nouhuys & Lei, 2004). Regarding the SP, the generalist parasitoids were apparently more efficient than the

specific *B. servadeii* in exploiting an abundant and fairly stable egg resource. Our results further evidence that the generalist parasitoids showed synchronized emergences with the male flight period of *T. pityocampa*, a fact which may be explained by the exploitation of alternative hosts. By contrast and as expected, the specialist parasitoid *B. servadeii* was well synchronized with the flight period of the winter population, but not with the reproductive period of the SP.

In general, *T. pityocampa* egg parasitoids prefer smaller clutches with larger eggs (Pérez-Contreras & Soler, 2004), suggesting that parasitism may also affect the trade-off between the number of eggs and egg size. Although higher parasitism could then be expected in Leiria SP, low rates of parasitism were instead observed. Nonetheless, this was also observed Leiria WP, indicating that this could be related to the abundance of parasitoids in the Leiria area. Nevertheless, in the present study a significant increase of parasitized eggs with the number of eggs per batch was detected, apparently contradicting the hypothesis that parasitoids prefer smaller clutches.

Conclusions

Present data demonstrate that the new Leiria SP exhibits a marked phenotypic divergence in relation to the sympatric WP from which the founders probably originated. Since the SP has recently evolved (Santos et 2007, 2011a), present results demonstrate that several traits (egg size / fecundity / egg covering) evolved rapidly, and that genetic diversity allowed for selection to act. In agreement, high intra-population phenotypic variability was found on the scale morphological traits (Fig. 6).

Rapid morphological differentiation and adaptation to local environments has been documented for species invading new habitats (Lambrinos, 2004). Here we document the occurrence of rapid differentiation of the phenotypic characteristics and life traits, for a population remaining *in situ*, using the same habitat and host plant, while having a shifted reproductive season.

Our data further agree with the hypothesis that divergence is probably speeded up and strengthened by exposure to the different ecological pressures now experienced by the SP (Santos *et al.*, 2011b). As a result, a tendency for a shift in the bionomic strategy of the SP is showed,

resulting from a higher energetic investment allocated to survival in relation to reproduction. This is in agreement with (Soberón, 2010), who identified bionomic effects as fundamental to explain species distribution at site and local scale. This work, therefore, provides a new insight of how ecological divergence may follow the process of allochronic reproductive isolation, a very rarely observed phenomena of sympatric speciation.

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5. SALTATIONAL SHIFT IN PHENOLOGY: EVIDENCE FROM AN ALLOCHRONIC POPULATION

Unpublished

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Saltational shift in phenology: evidence from an allochronic population

1. Introduction

Phenology determines the timing of life-cycle events of animal and plants. In a seasonally fluctuating environment, it is crucial for organisms to reproduce and grow in the right season, when climatic and food resources are most favourable. Such a fine temporal congruence of reproduction will allow individuals to pass on their genes to the next generations. Phenology is certainly a strong force shaping the evolution of species, particularly in temperate zones with contrasting seasons. Any mutation leading to a sudden phenological shift implying to reproduce in a different season, and consequently possibly leading to a mal-adaptation, might have a strong counter selection effect. Further, a shift in the phenology may ultimately lead to reproductive isolation by time as the shifted individuals will reproduce in a different season than the ancestral population, thus leading to allochronic speciation processes. Such processes have been recently documented in arthropods (Alexander & Bigelow, 1960; Cooley *et al.*, 2001; Teixeira & Polavarapu, 2003; Abbot & Withgott, 2004; Jankowski, 2004; Santos *et al.*, 2007; Yamamoto & Sota, 2012).

Climate is a major factor shaping phenology. Temperature in particular is considered the most basic controller of seasonality in poikilothermic organisms (Zaslavski, 1988; Bale et al., 2002) and a large body of literature exists relating the effects of temperature to developmental rates and phenology (Powell et al., 2000). In cold temperate regions, temperatures may determine a limited period in the year favourable for successful reproduction and growth, thus imposing univoltinism (Powell et al., 2000; Visser & Both, 2005).

Climate change has been proved to have a high impact on insect populations, either by shifting the range expansion northwards and upwards (Parmesan et al., 1999; Battisti, 2004; Hickling et al., 2006), or by affecting the phenology, not only in insects, but across taxonomic groups (Buxton, 1933; Dewar & Watt, 1992; McCarty, 2001; Bale *et al.*, 2002; Walther *et al.*, 2002; Visser & Both, 2005; Parmesan, 2006; Carstens & Knowles, 2007; Feehan *et al.*, 2009; Gordo & Sanz, 2010). Such phenological shifts could be interpreted as necessary adaptations to a new climatic scenario.

For primary consumers, such as phytophagous insects, synchrony with host plants is further crucial, to guarantee that the animal is active during the period when the food sources, such as plant tissues, are available, or more nutritive. Even minor seasonal changes in plant phenology, or in the quality of plant tissues, may affect body growth and fecundity substantially ((Buxton, 1990; Via, 1990; Felton, 1996; Ivashov et al., 2002; Coley et al., 2006; van Asch & Visser, 2007). This is particular crucial for those organisms that feed on highly seasonal food sources, such as buds, flower or fruits, for which the window of opportunity is narrow.

Finally, seasonal climatic variations also produce changes in the intensity of competition and predation. For those organisms which anticipate, or delay its activity in relation to competitors, or natural enemies, a competitor or enemy free space could result advantageous and compensate for a less favourable season (Maron et al., 2001; Van Nouhuys & Lei, 2004).

Shifts in phenology accompanying climate change are usually gradual, either by the anticipation of events in early spring or the delay of others in late autumn (Dewar & Watt, 1992; Dingemanse & Kalkman, 2008; Doi, 2008). Yet, a different scenario can be caused by mutational shifts. These shifts can happen suddenly, when random mutations occur in genes connected to phenology (Loxdale, 2010) so that the offspring is presented with a set of conditions completely different from those to which it was originally adapted. It is possible that such shifts happen in nature, but offspring survival is most likely hampered due to maladaptation, thus descendants cannot pass on the mutation to their offspring and found a new population. Still, avoiding competition or natural enemies might favour those that are born on a less suitable season, regarding climate or food sources.

Mediterranean areas are particular rich on variable phenology observed among close species which might be explained to the mild winter season. Regions with mild weather are probably favourable to mutational phenology shifts as the "wrong" season might not be so harsh. Shift from spring reproduction or development to the fall, or winter, are observed in some close animal species, such as, for example within the genus *Thaumetopoea*, where the phenology of some species vary across latitude (Démolin, 1969; Huchon & Demolin, 1971; Montoya & Robredo, 1972; Démolin *et al.*, 1994) or in the beetle genus *Tomicus* (Kennedy & McCullough, 2002; Gallego et al., 2004; Faccoli, 2005; Vasconcelos et al., 2006). Yet, in spite of such examples of variable phenology within the same genus, to the extent of our knowledge, observations in nature evidencing saltational evolution by phenological shifts are still lacking. In

this work we document a case where a phenological shift in a univoltine insect species, the pine processionary moth *Thaumetopoea pityocampa*, originated an allochronic speciation process from a founder population (Santos *et al.*, 2007; Santos *et al.*, 2011a). Based on laboratory studies of emergencies of the parent generation and cross mating, we searched for evidence of a saltational shift at the origin of a shift in phenology.

The pine processionary moth is a defoliator of pines, considered to be one of the most important pests in the Mediterranean basin. This insect has a univoltine life cycle, characterized by winter larval development. The harsh winter conditions are overcome by gregarious behaviour and by the spinning of dense silk nests, that allow larvae to maintain their temperature above lethal values (Battisti et al., 2005; Battisti et al., 2006).

Research is ongoing regarding a recently discovered case of allochronic speciation in Portugal (Pimentel, 2004; Santos, 2004; Pimentel *et al.*, 2006; Santos *et al.*, 2007; Santos *et al.*, 2011a). In *T. pityocampa* populations having a typical phenology (here forth named WP, for Winter Population), adults emerge in the summer and larvae develop throughout the winter. However, in the population with a shifted phenology (here forth named SP, for Summer Population), adults emerge in the spring and larvae develop throughout the summer. Adult flight was monitored over five years, revealing a gap of 15 to 27 days between the flight periods of the two populations, and since adults live for about three to four days only, reproductive isolation is assumed (Santos *et al.*, 2007). Ecological implications of this phenological shift are mostly related to temperature and host quality during larval development. SP larvae do not have to endure low winter temperatures, which allows them to stay out of the nests and feed for longer periods, consequently investing less energy in spinning dense silks nests. Regarding heat conservation, the summer phenology thus appears advantageous. Yet, other ecological factors might be adverse. For example, extreme hot temperatures in the summer could pose survival difficulties for SP larvae, as according to Démolin, (1969) the upper threshold of tolerance for the young larvae would be 32ºC. It could be inferred that a much higher threshold for larval tolerance to temperature would have evolved in the SP, as demonstrated by Santos et al. (2011b).

Previous genetic and ecological research has shown that this atypical population is genetically differentiated from the sympatric population (Santos *et al.*, 2007; Santos *et al.*, 2011a). Divergence in ecological aspects such as the temperature tolerance in young larvae, as

well as differences in the egg stage, female fecundity and pressure from natural enemies have been described (Pimentel et al., 2012; Santos et al., submitted) .

Climate change, such as warmer summers and winter seasons, which would allow individuals to pupate and emerge earlier, might be suggested as an origin for the shifted phenology. An alternative hypothesis would be that a saltational shift in one or few individuals would have occurred, due to a mutation in one or more genes, such as those involved in the diapause. To investigate this hypothesis, we here propose to study under laboratory conditions the phenology patterns of adult emergence over several years, both in the two parental populations (Leiria SP and WP) and their F1 hybrids, aiming at determining the phenotypic heritability of the emergence pattern. To obtain hybrids, pupal diapause was manipulated in the laboratory through temperature, so that an artificial synchronization of adult emergence of the two sympatric populations was achieved. Since the level of genetic differentiation between the two parental populations is high (Santos et al. 2011), a further objective was to test the possibility to identity hybrids by microsatellite genotyping, which could further be used to test for a probable presence of hybrids in the field.

2. Material and methods

2.1. Sites

Individuals of the two populations studied, SP and WP, were collected in Leiria Portugal (39º 50'N; 08º 55'W altitude 20- 50m), where the two populations are sympatric. As an external population, used for comparison, we also sampled a third population, namely Apostiça (38º 30'N; 09º 11'W, altitude 35m), located ca. 100 kms in the south Mata Nacional de Leiria,.All areas studied are stands of maritime pine.

2.2. Survey of adult male emergence in the field

Flight monitoring in the field was conducted by capturing males with funnel traps baited with the female synthetic sex pheromone (Z)-13-hexadecen-ll-ynyl acetate (Guerrero et al., 1981) (Pityolure, 40mg, AGRISENSE). Monitoring took place in the area of occurrence of the SP and

in proximal areas located up to a distance of 20 km, as well as in Apostiça. The flight period was surveyed over four years (2005, 2008, 2009 and 2010) in Leiria and three years (2004, 2005, 2008) in Apostiça. In each year, four to 14 traps were installed in April and checked either weekly, or every two weeks, until the end of September.

2.3. Emergences and cross-matings in the lab

Adult emergences in the laboratory were obtained by collecting mature larvae, L5 ready to pupate, or pupae in the field (fig 1 a). SP pupae were collected from the ground in December/January, when WP was in the larval stage inside the nests. WP was collected as L5 in February by colleting larval nests from tree canopy, just prior to pupation. In the laboratory the larval nests were placed inside plastic boxes and fed with maritime pine until pupation. About one month after, to ensure the pupation was completed, pupae were then removed from the sand. All pupae were exposed by removing the cocoon, sexed and counted (Fig 1 b). Healthy pupae were re-counted, and separated into groups that would be exposed to different temperatures until adult emergence.

Collections were made between 2008 and 2011. Each year, about 1000-1300 healthy pupae of both SP and WP populations were obtained. Emergence rates varied between 27 and 97% for the SP, and between 60% and 98% for Leiria WP. For the pupae, the main factor of mortality was parasitism.

Since no previous studies for this shifted population existed, in the first year of the project several temperatures had to be tested in order to delay SP emergence and obtain SP adults by the time of WP emergence. Temperatures of 12ºC, 13ºC and 15ºC were tried to delay adult emergence. At 15ºC the rate of emergence was high (75%). Yet, the period of emergence at this temperature, from the 18th of April to the 2nd of June, was only delayed by one to two weeks, in comparison with the individuals kept at room temperature (22ºC ±2) and did not coincide with the emergence of WP adults. As for 12ºC, only 2% of the adults emerged, and most individuals presented malformations such as lack of muscular tonus, or even incapacity to molt, fly or mate (Fig 1 e and f).

Figure 1 - Collection of pupae and crosses. a - pupae collected in the field; b - pupae separated by sex, after removing the outer cocoon; c - adults mating; d - female laying eggs; e - pupae kept at 12ºC with adult unable to completely emerge; f – malformed female, from pupae kept at 12ºC.

Observed symptoms were inability to emerge from the puparium, to fully spread the wings or fly, inability to grip to the needles, or any other substrate, apparent lack of strenght in the legs. Similar symptoms were also observed at 13ºC for a lower percentage of insects and were plausibly caused by the prolonged exposure to below optimal temperatures in the post-diapause

morphogenesis period (e.g. (Williams, 1956; Tauber & Tauber, 1976). These adults failed to reproduce.

It was concluded that 13ºC was the less damaging temperature for the pupae and it was thus used for delaying SP development.

Crossings were performed in laboratory. In the first year of the experiments, pupae were kept in individual plastic vials and pairs formed as the adults emerged. However, a low rate of mating was observed, due to several factors, including adult manipulation. Later in the same year, pupae were separated by sex and placed in larger chambers with good ventilation. As individuals were separated by population and sex, one chamber per cross type with the respective males and females was prepared, allowing adults to emerge directly into the chamber, without further manipulation being required. Chambers were large enough for adults to fly, and open windows allowed for air to circulate inside and pheromone plumes to form.

Six types of crosses were performed (always named after [female origin]*[male origin]): 1) SP*SP, Leiria SP during their normal period of emergence (May); 2) SPd*SPd, within Leiria SP with delayed parent emergences; 3) SPd*WP, delayed female from Leiria SP, male from Leiria WP; 4) WP*SPd, female from Leiria WP, delayed male from Leiria SP; 5) WP*WP, within Leiria WP, during their normal period of emergence; 6) WPAp*SPd, female from Apostiça, delayed male from Leiria SP.

For egg laying, fresh branches of pine were placed inside the chambers, and changed every two or three days. Additionally, a set of wooden toothpicks and cocktail sticks inserted in a Styrofoam base, were placed in the chambers, to act as a second choice for female oviposition. Most egg batches were collected both from the pine needles and the wooden sticks and toothpicks, but some females laid their eggs in flat surfaces, like on the wooden structures of the chambers, on the chamber walls, on the Styrofoam bases, on the paper covering the floor of the chamber or on the pine branches.

2.4. Larval rearing

Resulting F1 eggs of the 6 types of crosses were preserved at laboratory conditions, until the larvae hatched. Once the young larvae hatched, they were separated by family (egg-batch) in acrylic boxes and fed with fresh maritime pine needles attached to branches, three times a week

until pupation. Boxes were cleaned weekly and mortality and development was reccorded. Once larvae were in the 5th instar, the boxes were filled with a layer of approximately 10 cm of sand, to allow burrying and facilitate pupation. Sand was collected in the field and passed trough a sieve, to ensure that no pupae from the field, or other insects were brought into the boxes.

About one month after all remaining larvae burried themselves in the soil, all boxes were emptied from the sand and sieved to collect pupae. Pupae were then stripped from their outer cocoon, counted and sexed. Each family was kept in a separate container. Emergences of F1 adults were registered and the adults kept in absolute alcohol for genetic analyses. Temperatures inside the laboratory were recorded with a datalogger.

2.5. Statistical analysis

Generalized linear model (GLM) was used to model adult emergences in the laboratory from the pupae collected in the field, considering two factors, population and year, nested within population. Normal distribution and maximum likelihood estimates were used.

To decompose the sources of phenotypic variance of the offspring emergence dates, a linear mixed model (LMM) was fitted with the form:

$$P_{ij} = \mu + C_i + F_j + \varepsilon_{ij}$$

Where P represents the phenotypic individual observation; μ is the overall mean; C_i, the effect of ith type of mating-cross (fixed); F_j, the effect of jth parent (random) and ε, the residual error. Variance components for parent (σ^2_p) and residual errors (σ^2_ε), with the respective associated standard errors, were estimated by restricted maximum likelihood (REML). Singularity matrix was used for random components.

Estimate of broad-sense heritability (H^2) was calculated from the components of variance as the fraction of phenotypic variance that is not due to environmental effects ($H^2 = \sigma^2_p/\sigma^2_{Total}$) (Lynch & Walsh, 1998), where σ^2_p represents the genetic variance due to parental families and σ^2_{Total} is the phenotypic variance plus the error term.

Estimates of fixed effect, mating-cross types, were obtained and compared pairwise by Least Square Deviance (LSD).

Since the days of emergence for both mother and offspring were known, as well as the phenotypic varability for both generations, narrow-sense heritability (h^2) was also estimated (Lynch & Walsh, 1998):

$$h^2 = \frac{2Cov(P,O)}{Var(P)} = 2b \qquad (2)$$

Where *Cov(P, O)* represents the covariance parents-offspring, *Var(P)* represents total phenotypic variance of parents, *b* is the regression coefficient of offspring emergence on parents emergence day.

SPSS software 18.0 was used for the statistical analysis.

2.6. Genetic analysis

To ensure the genetic identity of the hybrids obtained, and to test our capacity to identify hydrids using microsatellite data, we used one larva per offspring from nine crosses [female WP x male SP] and 13 crosses [male WP x female SP] performed in 2009. Since the parents were not available for genetic analysis, we used as reference populations 30 SP larvae collected in 2007 and 36 WP larvae collected in 2004. It was already demonstrated that the genetic structure of each population was stable through different years of sampling (Santos et al. 2011a) and we then considered that those samplings are representative of the parental populations.

Nineteen microsatellite loci are available for *T. pityocampa* (Rousselet *et al.*, 2004; Santos *et al.*, 2007; Burban *et al.*, 2012). We used 17 of them, i.e. removing MS-Thpit6, located on the sexual chromosome, and therefore not useful to discriminate hybrids, and MS-Thpit2 that exhibits a large amount of null alleles in the winter population. Details of DNA extraction, amplification and molecular protocols for genotyping are described in Rousselet et al. 2004 and Burban et al. 2012.

Individual assignment was realized using Bayesian inference implemented in Structure 2.2.3 (Pritchard *et al.*, 2000), using the same parameters as Santos et al. (2011) achieved for a larger sampling but with only 6 microsatellite loci (number of cluster K=2, 100 000 burn-in steps followed by 100 000 MCMC simulation steps with a model allowing admixture). We also

performed Bayesian model-based clustering analysis with NewHybrids (Anderson & Thompson, 2002), using Jeffrey-like priors, and 100 000 burn-in steps followed by 100 000 MCMC simulation steps. It allows for each individual, without any prior information, to compute the posterior probabilities that it belongs to parental or to F1 hybrid clusters.

3. Results

3.1. Adult male flight patterns

Male catches with pheromone traps in the field evidenced distinct flight seasons for Leiria SP when compared with the two winter populations, Apostiça and Leiria WP. Moreover, these two winter populations also differed, Leiria WP showing earlier flight activity (Fig. 2).

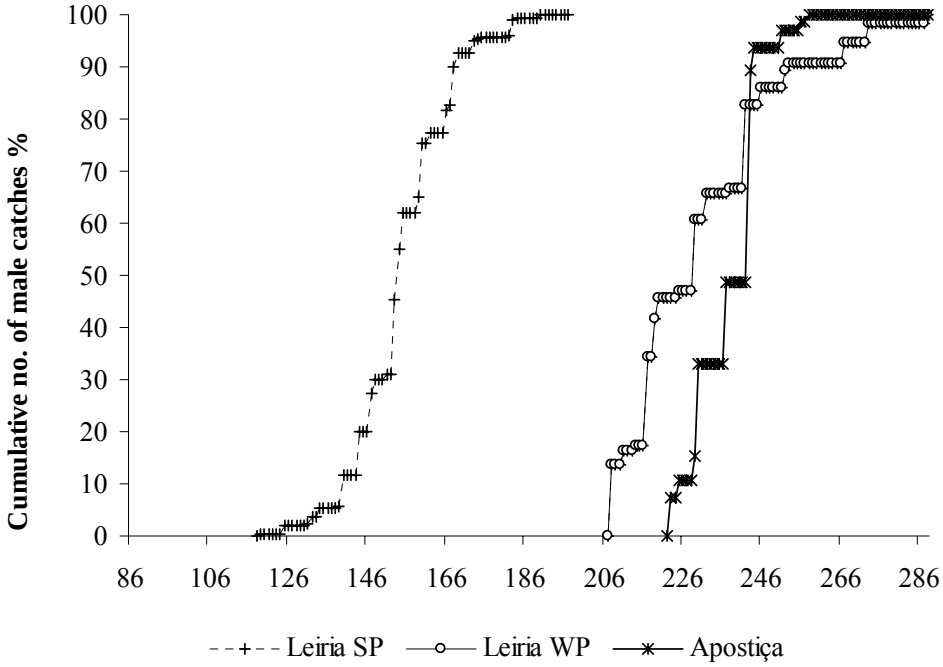

Fig.2. Male catches with pheromone traps for three *Thaumetopoea pityocampa* populations, Leiria SP, Leiria WP and Apostiça, expressed as cumulative mean percentages (+SE). Field monitoring was conducted in 2005, 2007, 2008, 2009 and 2010 in Leiria and in 2004, 2005 and 2008 in Apostiça.

3.2. Emergences in the laboratory of field collected pupae and larvae

The emergence of SP adults could be successfully delayed in the three years of experiments. As a result, the periods of emergence of the adults of the two populations overlapped and cross-mating was possible with insects kept at 13ºC during the pupal stage. However, each year the first adults that emerged always presented malformations and could not mate.

Different types of crosses were successfully achieved. For SP x SP, two types of crosses were obtained, one called SP x SP, using non-manipulated insects that emerged during the normal reproductive season, and another with adults resulting from delayed emergences, named SPd x SPd (Table 1). Necessarily all hybrids resulted from SP delayed individuals.

A total of 2134, 694 and 2845 adults emerged in the laboratory, from pupae/mature larvae collected respectively in Apostiça, Leiria SP and Leiria WP. GLM evidenced significant differences between populations, with Wald Chi-square = 137151.3, df = 2, $p < 0.001$. From pairwise comparisons it is concluded that all populations differ significantly among them, Leiria SP showing the earliest emergences (112 ± 0.23), Leiria WP intermediate ones (199 ± 0.15) and Apostiça the latest emergences (225 ± 0.25).

Within each population, significant differences were further found among years, Wald Chi-square = 4628.8, df = 7, $p < 0.001$. Although a similar pattern of emergences was observed for all populations over the years, variations of the mean time of emergence were observed between years (Fig. 3 and 4).

Emergences in the laboratory anticipated field emergences for the SP by approximately 36, 21 and 13 days, respectively in 2008, 2009 and 2010, and for the WP by 29, 31 and 35 days, respectively in 2008, 2009 and 2010.

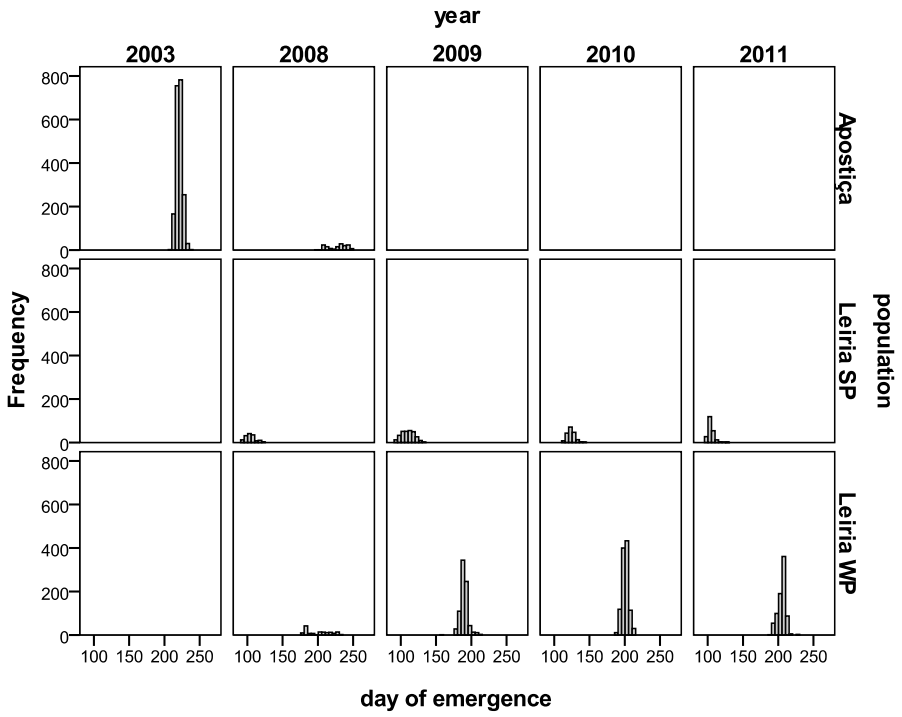

Fig. 3 – Histogram of adult emergences obtained in the laboratory from pupae / mature larvae collected in the field during late winter.

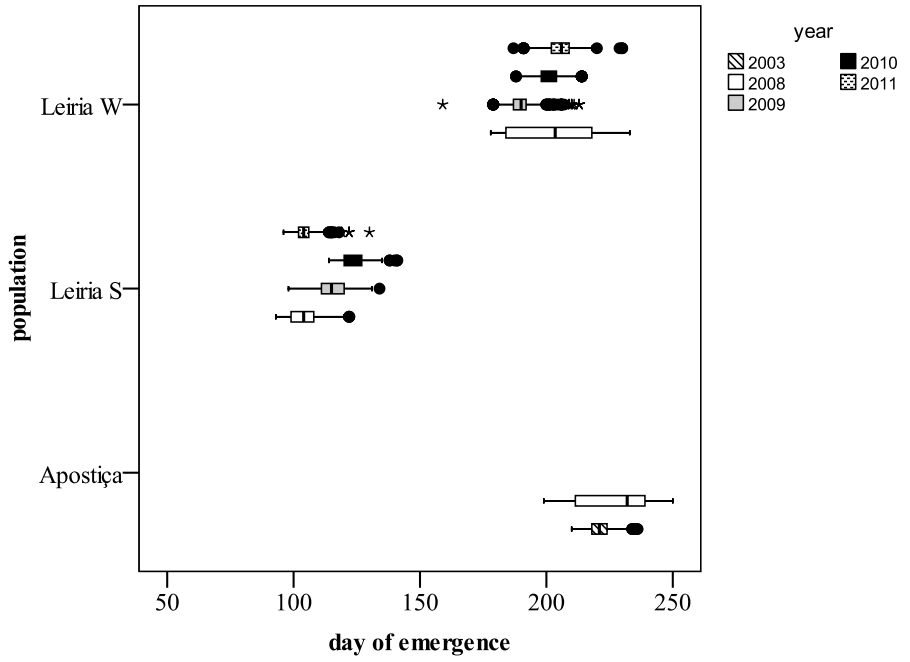

Fig. 4 – Histogram of adult emergences in the laboratory obtained from pupae / mature larvae collected in the field, in late winter.

Prolonged diapause occurred in SP pupae collected in the field in 2008 and 2011, emerging in the laboratory respectivelly in 2009 and 2012. However, it occurred only for pupae that were submitted to low temperatures (12ºC, 13ºC and 15ºC) to obtain delayed adult emergences, in 1.2% to 3.3% of the pupae. Emergences of individuals with prolonged diapause occurred with two weeks delay in comparison with SP individuals without prolonged diapause, that emerged in 2008 and with a four days delay, when compared to SP individuals collected and emerged in 2009.

3.2 Cross-matings

Different types of crosses were successfully achieved. The numbers of eggmasses obtained per type of cross are given in Table 1.

Table 1. Total number of egg batches (representing successful matings) obtained for each type of cross-mating, per year.

Cross type		**Number of eggmasses**			
female	**male**	**2008**	**2009**	**2010**	**2011**
SP	SP	9	19	43	46
SPd	SPd	2	23	3	0
WP Leiria	SPd	8	15	21	70
WP Apostiça	SPd	4	n/a	n/a	n/a
SPd	WPLeiria	7	19	3	2
SPd	WP Apostiça	3	n/a	n/a	n/a
WP	WP	15	186	45	50

From the six types of crosses, 501 adults emerged in the laboratory from 27 full-sib families, in 2009 and 2011 from crosses obtained in 2008 and 2010, respectively. The period of emergence differed according to the type of crosses (Fig. 5 and 6).

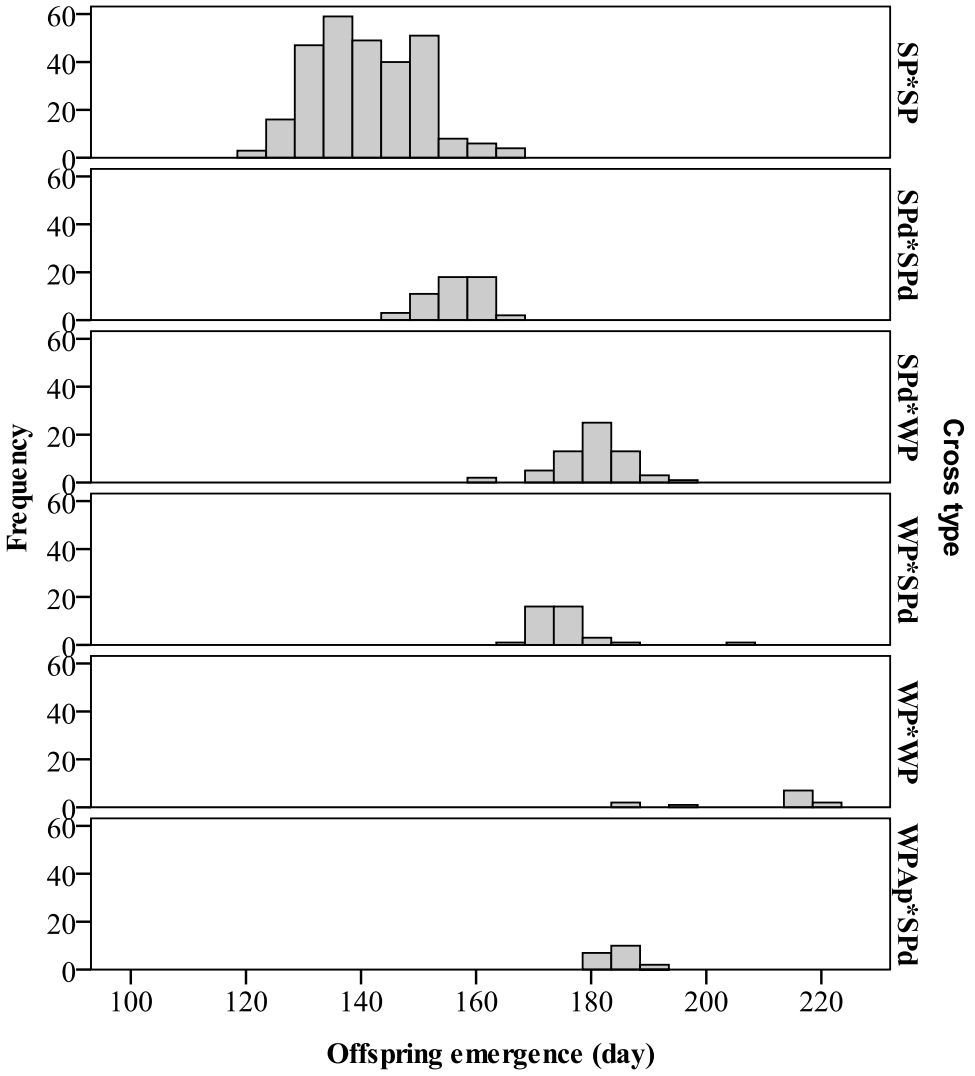

Fig. 5 – Histogram of offspring emergence obtained from matings conducted in the laboratory in the previous year for the six types of crosses performed.

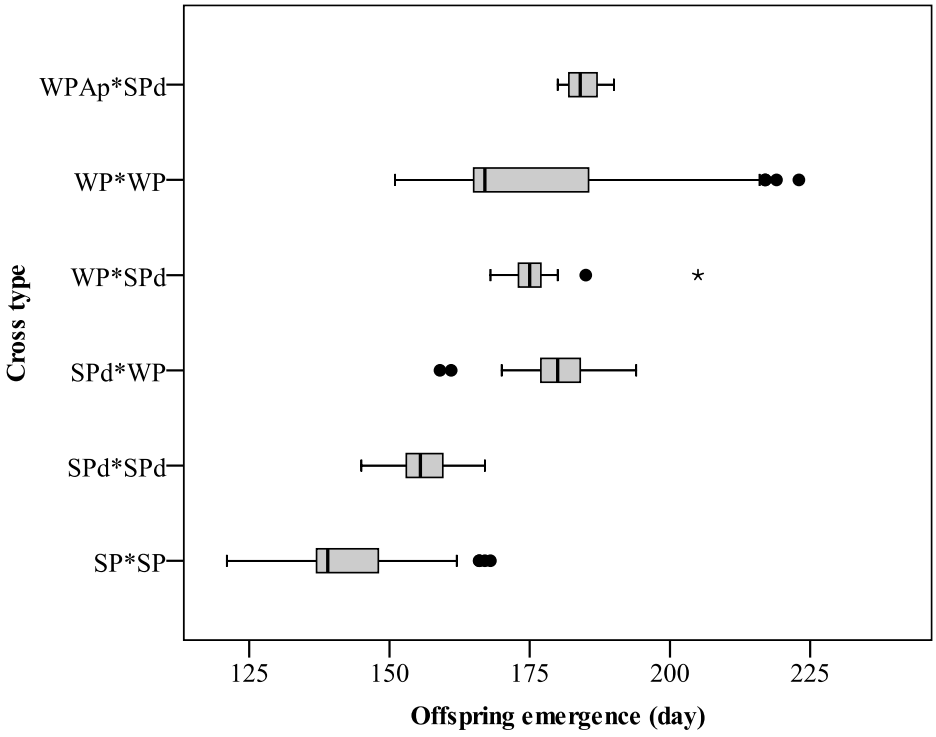

Fig. 6 – Box-plots of offspring emergence obtained from matings conducted in the laboratory in the previous year for the six types of crosses performed.

3.3. Cross type effect and variance components

Developmental time, i.e. the number of days from oviposition until adult emergence, was highest for SP*SP crosses, being significantly higher than one year (365 days) 95% CI [374-392], which means the development took ca. 2-3 more weeks compared to the to the cycle observed in the field. Developmental time for both WP*WP and WPAp*SPd fall within the expected one-year cycle 95% CI [347-361] and [309-370], respectively. Yet, developmental time was significantly lower than the one-year cycle for all other crosses, and especially for the SPd*SPd crosses, for which the emergence of the parents was delayed in the laboratory (Fig. 7). Consequently, the resulting offspring, from parents with delayed emergence, emerged in the following year close to the date of emergence of the SP*SP offspring, obtained from parents having emerged in the laboratory without any manipulation of pupal temperatures, Both types of offsprings did not differ significantly from each other regarding the date of emergence (Fig. 8).

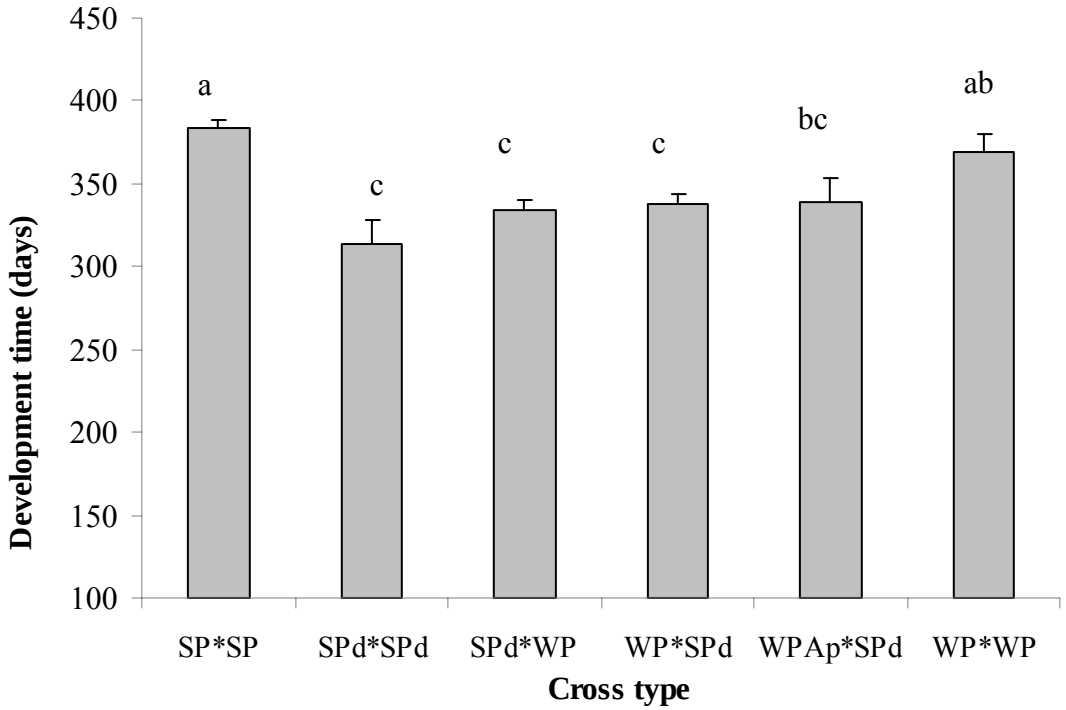

Fig. 7 – Mean time of development in days (+SE), obtained by General Mixed Model, of offspring from the six cross types performed

Offspring from SP*SP occurred earlier than expected. Moreover, their dates of emergence differed significantly from all types of crosses, except from SPd*SPd, as mentioned above. Hybrids showed intermediate values and WP*WP adults were the last ones to emerge, as expected.

Variance components estimated by LMM, allowed for the estimation of a 95% confidence interval (CI), for broad-sense heritability H^2 of the emergence day to be [0.647-0.897].

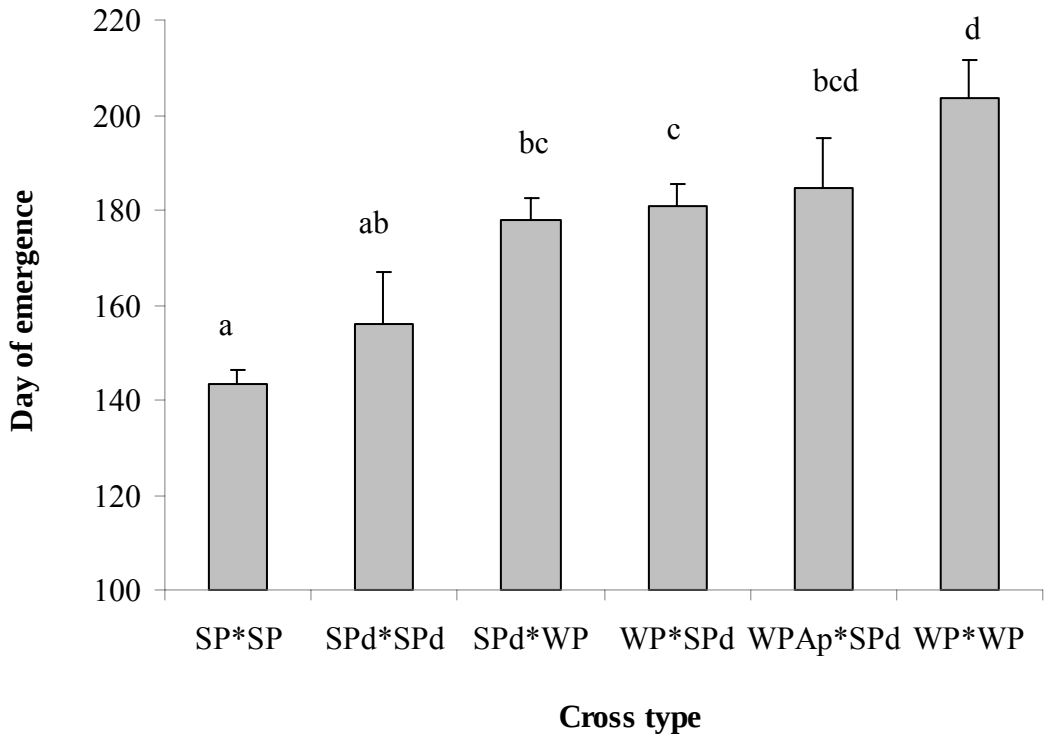

Fig. 8 – Mean estimate (+SE), obtained by General Mixed Model, of offspring from the six cross types performed

3.4. Narrow sense heritability

When considering all types of crosses, from the regression of offspring phenotypic values on the parental phenotypic values, 95% CI for narrow sense heritability h^2 is estimated to be [0.730 - 0.844]. If SPd*SPd are excluded from the regression, considering both parents were manipulated by cold treatment to emerge later, 95% CI for narrow sense heritability h^2 is then estimated to be [0.874 - 0.986].

3.5. Genetic analysis

All individuals were successfully genotyped for the 17 loci. Graphical summary of assignments of the 82 genotyped individuals is provided in Fig. 8. Using Structure software, a

membership coefficient threshold set to Q = 0.80 resulted in 100% of the reference individuals being correctly assigned to their "phenological" phenotype. All experimental hybrids had intermediate assignment coefficients between the two clusters, except for one individual (number 86) assigned to the phenological winter population (Q=0.96). However, hybrid assignment scores ranged from Q(SP-WP)= 0.23-0.78 to Q= 0.48-0.52.

NewHybrids pertinently assigned individuals of each parental reference population to their respective cluster with a posterior probability threshold (equivalent to the membership coefficient Q computed in Structure) qn=0.99. Using the same threshold, all experimental hybrids, except two, were assigned to the genetic F1 cluster. The individual number 86 is totally assigned to the parental cluster of the phenological winter population. Another individual (number 67), although mainly assigned as F1 hybrid had a probability qn=0.06 to belong to the parental WP cluster.

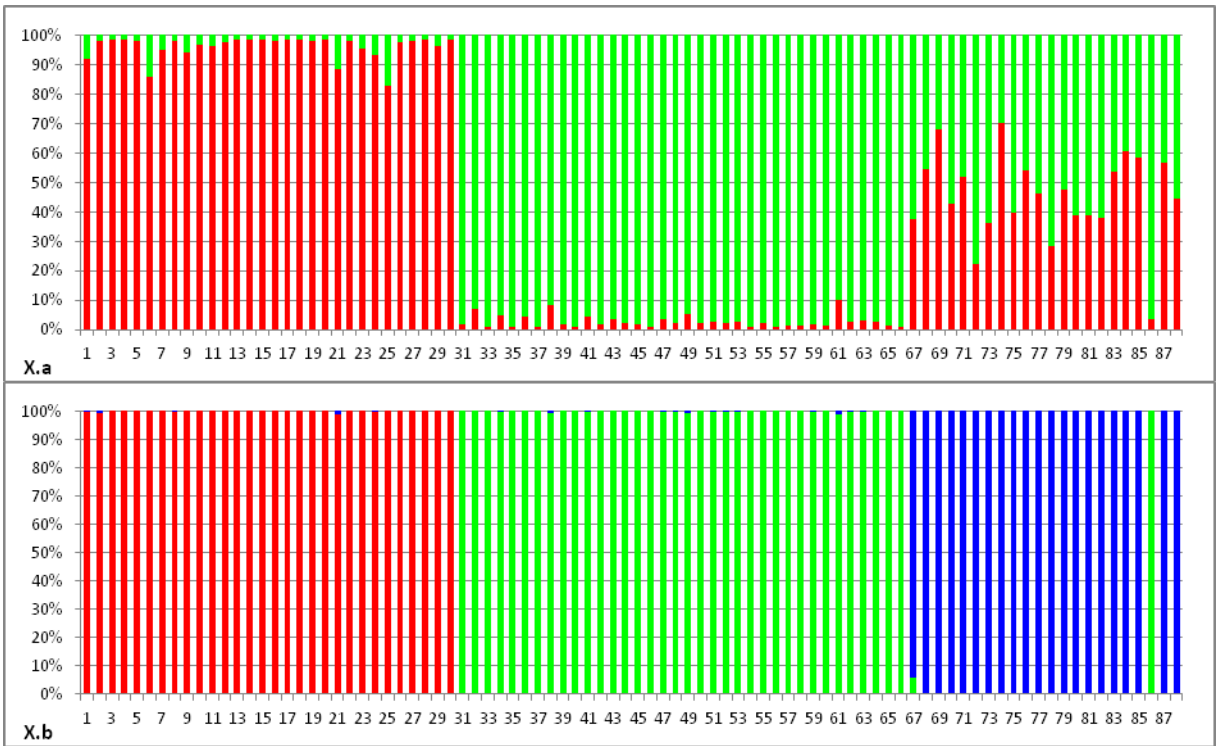

Fig.8. Graphical representation of the individual assignation of SP parental population (1-30), WP parental population (31-66), experimental offspring [female WP x male SPd] (67-75) and [male WP x female SPd] (76-88), genotyped with 17 microstellite loci. A Structure Q-matrix obtained with for K=2. X.b NewHybrids qn probability to belong to one or the other parental genetic class (red and green) and F1 hybrid class (blue).

4. Discussion

Population and between years variation

Flight phenology follows the same pattern of emergence within each population, SP, WP and Apostiça. Nonetheless due to natural climatic inter-annual variations, it is expected that variations in phenology also occur within populations, according to environmental conditions, especially temperature (Visser et al., 2010). Temperature not only affects development, but is also crucial in the process of diapause, especially regarding its termination (Gilbert & Raworth, 1996; Hodek & Hodková, 1998; Denlinger, 2002).

It is also very interesting to observe that, in spite the adult emergence was artificially delayed, offspring resulting from these adults emerged at the expected date for the non-delayed parental populationrepresenting a shorter cycle, which indicates that the phenology is not completely altered by temperature alone. Individuals resulting from SPd crosses experience a shorter life cycle where the pupal stage is the most affected.

Phenology of hybrid offspring

The emergence of the adult hybrids reveals intermediate behaviour when compared to the parental populations. The flight phenology of the hybrids does not match those of the parental populations, and there are no signs of dominance of the parental phenology. It can thus be conclude that this is a co-dominant trait and that flight patterns were consistent throughout the years of experiments.

This fact strengthens the hypothesis of a saltational shift, with very distinct flight phenology, and hence, reproductive period, probably caused by a random mutation in a gene linked to phenology, or to the termination of diapause.

Hybrid genetics

The strength of the set of markers we used proved to pertinently assign SP and WP individuals to their respective phenological population, using both methods tested. However, NewHybrids allowed a correct assignation with a higher threshold than Structure. One can note

that for the same individuals, the previous set of six microsatellites (Santos et al. 2011) loci gave ambiguous assignations for five out 66 parental individuals genotyped here.

NewHybrids seems also more accurate and efficient in hybrid detection, as all except two individuals were assigned as F1 hybrids with a probability qn > 0.99.

With both methods, one experimental hybrid is surprisingly assigned to the parental cluster having winter population phenology. We cannot exclude the possibility that its presupposed genetically summer mother might instead have belonged to the winter population, for its genetic neutral background studied here, in spite of exhibing an aberrant summer phenology. Similarly, the occurrence of a new mutation was hypothesized by Santos et al. (2011) at the foundation of the summer population, to explain the summer phenology. However, a misidentification of this individual seems highly plausible, as such a combination of WP genotype - SP phenology is not found in natural populations.

The second case of an individual not completely assigned as F1 hybrid with NewHybrids, may be more informative. It has been here demonstrated that F1 hybrids exhibited, in the laboratory, an intermediate phenology. If such crosses could happen in nature, then rare back-crosses with the parental winter population could result in a relatively early winter phenology. Indeed, to succeed laboratory crosses, even if the emergence of the summer parents were artificially delayed, we certainly selected parents exhibiting the latest dates of emergence among the summer population and the earliest dates among the winter population. We therefore maximized the probability to use parents having an hybrid in their own pedigree (in other words, parents for which the genome is partially introgressed by the sympatric SP), as far as such hybrids might exist in natural populations and considering that intermediate phenology would be quantitatively heritable over generations. However, this result could also illustrate the limits of the detection power of the markers used.

Analysis of larger samples will be necessary to look for hybrids in natural populations. Simulated genotypes could also be used to establish the level of discrimination possible, using the two methods proposed, between closed classes of genotypes, including backcrosses.

Conclusions

We here evidence the occurrence of a phenological shift that could have led to a rarely documented saltational evolution, in spite of gradualism being generally accepted as a model for evolution (Rubinoff & Le Roux, 2008; Theißen, 2009). Furthermore, a discontinuous evolutionary model has been documented for pheromone evolution (Baker, 2002; Roelofs et al., 2002; Symonds & Elgar, 2008), as well as particular traits in arthropods such as segment number in centipedes (Minelli et al., 2009), or Batesian mimicry, host plant shift, and physiological differences in light of phylogenetic data in sphynx moth (Rubinoff & Le Roux, 2008). This could represent a unique opportunity to study a case of ongoing allochronic speciation due to a saltational shift in phenology.

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6. CONCLUSIONS

6. Conclusions

6.1. Genetic analyses

Allochronic speciation followed by ecological differentiation has rarely been observed and studied. The case study ilustrated by the pine processionary moth is thus unique and valuable, – since it contributes to improve present knowledge of this particular evolutionary process. With this work, an understanding of the genetic trends of differentiation unfolding during this early evolutionary process was achieved. A continued observation of the population in the future will further contribute to highlight the genetic and ecological evolution of this reproductively isolated population.

Previous results, based on mitochondrial and nuclear sequences revealed that the SP belongs to the same species as the surrounding WPs (*T. pityocampa*), suggesting that allochronic differentiation was on-going, as a result of the phenological shift (Santos et al., 2007c). Moreover, microsatellite markers revealed a strong differentiation between SP and WP, with signs of a founder event in the SP.

In this study, we aimed at analysing the genetic structure of the two sympatric populations, assessing the stability in time of the population showing a shifted phenology, finding signs of hybridization and testing the stability of the genetic structure of both populations.

From the genetic sampling and analysis of larvae and adults of *T. pityocampa*, mostly from Leiria, that were genotyped using 6 microsatellite markers, we can state that there is both a clear and extreme differentiation between SP and WP, and a stability in time of the genetic structure observed in Leiria. The pairwise F_{ST} matrix, the PCA and the Bayesian analyses run with STRUCTURE all show that the SP is significantly differentiated from all WPs studied. Thus, our main conclusion is that the Leiria SP forms a very distinct genetic cluster, which is highly stable in time (Santos et al., 2011a).

Results on male captures in the field alone do not rule out the hypothesis that genetic exchange still occurs between populations. The dates of adult emergence are determined by the termination of the obligate diapause that the insect undergoes during the pupal stage (Huchon &

Démolin, 1970b), highly dependent on temperature and photoperiod (Gilbert & Raworth, 1996; Denlinger, 2002). Gene flow between both populations could thus occur via shifting of some individuals from one phenology to the other through the mechanisms underlying adult emergence. In other words, some "winter larvae" could emerge as "summer adults" after pupal diapause, and vice-versa. Should this be case, genetic evidence of gene flow between populations should be found, and we could expect the within-population genetic structure to vary in time, as a consequence of gene flow. The genetic data corroborate the reproductive isolation hypothesis; yet, we could detect a number of individuals for which genetic and phenotypic assignment were not consistent, which suggest that gene flow could occur between both populations (see below).

It can also be suggested that Leiria SP could now be considered as a highly differentiated "phenological race", in the same manner that host races have been proposed for phytophagous or parasitoid insects' lineages using different hosts (e.g. Dres & Mallet, 2002; Blair et al., 2005; Stireman et al., 2005; Stireman et al., 2006; Peccoud et al., 2009).

Different scenarios can be proposed to explain the existence of a PPM population with a shifted phenology. Based on previous results (Santos *et al.* (2007a), a scenario involving a recent local origin of the summer population from a sudden phenological "mutation" and a founder effect was proposed.

The genetic data presented here are mostly consistent with this hypothesis, even though one of the microsatellite markers, MS-*Thpit*2, gave rise to some doubts and needs further research. Except for this marker, we observed a lower number of alleles in the SP than in the WP for all sampling seasons, with all alleles found in the SP also present in Leiria WP (but not the opposite), and a distortion of the allelic frequencies.

Over five years of male flight monitoring, a bimodal curve of adult emergence in Leiria was always observed, whereas for *T. pityocampa* typical curves of male trapping are unimodal (Démolin, 1969b). A time lag of three weeks to one month was consistently observed around July. Our data strongly suggest that such a time lag occurs each year, between the latest emergences of the SP and the earliest emergences of the sympatric WP. As adults do not feed, and live up to a maximum of 3-4 days (Démolin, 1969b, H. Santos, ISA, Lisbon, pers. obs.), we could conclude that SP and WP individuals cannot mate, and that sexual reproduction between

the two sympatric populations is limited, or even non-existent. Phenology effectively leads to pre-zygotic isolation.

Nonetheless, results of a Bayesian analysis showed that a few individuals with WP phenology were genetically assigned to the SP, revealing some few cases of inconsistency between genetics and phenotypic identifications. We hypothesize that individuals from the SP can occasionally experience a longer diapause, and emerge as adults in July, or August, rather than April or May. They can then reproduce with WP individuals, causing a limited amount of gene flow between populations. This is probably a rare event, as it was never observed under laboratory conditions (MB & HS, pers. obs.). This would explain both the existence of "phenologically WP but genetically SP" males, and the occurrence of larvae that are identified by STRUCTURE as genetic hybrids.

Individuals from the SP are subjected to different ecological constrains and now experience unique selective pressures, as compared to other PPM populations. In particular, temperature and hygrometry during metamorphosis, embryogenesis and the first stages of larval development, as well as the quality of the foliage consumed and natural enemies, differ between those of the ancestral population. It is very likely that natural selection will lead to the evolution of adaptations to these peculiar conditions. This will further lead to reinforcement of the genetic differentiation. Allochronic speciation has been hypothesized in a few cases, as a mechanism to explain a past speciation event between fully separated taxa (Cooley et al., 2001b; Ritchie, 2001a; Abbot & Withgott, 2004c; Danley et al., 2007). We discovered here an exceptional case of on-going allochronic differentiation that could be seen as a first stage of an incipient sympatric speciation, without host shift.

6.2. Ecological adaptations

To begin assessing the ecological adaptations occurring in the SP we first worked with the more exposed life stage, that is the larval stage. Larvae of the SP develop throughout the summer, and even though the area where they occur has fairly cool summers, it is not rare that temperatures above 32ºC are registered, which corresponds to the upper temperature threshold proposed by Démolin (Démolin, 1969a). To clarify this inconsistency, we conducted experiments

to characterize larval survival at very high temperatures, for the two sympatric populations plus one WP from Bordeaux, used as control.

Larval survival differed significantly among the three populations tested, regarding tolerance to high temperatures. Tolerance was highest for Leiria SP and lowest for Leiria WP. Results are in accordance with the range of temperatures recorded locally, at the time of larval development, for each population and region. L1 larvae from Leiria SP are further subjected to maximum temperatures exceeding 35.0ºC, a temperature to which no other population from *T. pityocampa*, or from its closely related *T. wilkinsoni* is normally exposed. Thus, we conclude that due to the abrupt phenological change (Santos *et al*., 2007; 2011a), the early larval instars of this summer population (SP) were also suddenly subjected to considerably higher temperatures than before. Results further suggest that thermal tolerance was quickly acquired by a cohort of *T. pityocampa* SP founding individuals, from which enough survived to secure population viability. In consequence, descendents of such individuals are presently better adapted to cope with higher temperatures.

Although the mechanisms underlying these adaptations are not completely claryfied, within population variability, tolerance to high temperatures is a trait that would allow rapid evolution under strong evolutionary constraints to occur (Thuiller et al., 2005). We can hypothesize that physiological adaptations might be involved, such as divergence in the genetic mechanisms of regulation of heat-shock and stress-related genes, as observed in other insects (Mahadav et al., 2009).

Results also suggest that adaptation to even hotter summers can be expected and that temperature constraints might not preclude SP expansion beyond coastal areas, into more continental or Mediterranean regions.

Divergence of the egg traits between SP and WP was also analysed. A trade-off between egg size and fecundity is well established in the literature (e.g. (Fox & Czesak, 2000; Fischer & Fiedler, 2001; Gillooly et al., 2002; Fischer et al., 2003; Pérez-Contreras & Soler, 2004; Pimentel et al., 2010). The latitudinal gradient described for the pine processionary moth (Pimentel et al., 2010) suggests that higher egg size occurs in the southern parts of the distribution range, where

fewer eggs tend to be laid per egg batch. Early larval survival is favoured by increased egg/neonate larval size (Fox et al., 1997; Fox & Czesak, 2000).

The harsh winter temperatures in the northern range impose a high pressure, and due to the gregarious larval behaviour of *T. pityocampa*, the number of eggs in an egg batch will have a direct influence on colony size and survival (Pérez-Contreras et al., 2003). In colder areas this constraint will favour a higher number of eggs in disadvantage of larger neonates. A similar trade-off was observed in the present study for the shifted SP population, by comparison with other eight populations having winter phenology. Since SP larvae develop in the summer and face extreme hot temperatures, larval nests became looser structures with a smaller number of larvae (authors pers. obs.). Thus, lower fecundity with larger eggs is most probably advantageous. In contrast, summer hot temperatures may act as a limiting factor for the survival of SP larvae, as evidenced in a previous study (Santos et al., 2011b). Moreover, the SP larvae feed on a host with particularly tough needles, the maritime pine *P. pinaster* for which the size of the neonate larvae is critical (Zovi et al., 2008). This ecological factor will further reinforce the evolution towards fewer and larger eggs with more reserves, a trait that might improve larval performance.

Concerning the scales covering the eggs, a large variability within and among *T. pityocampa* populations was observed. In Leiria SP, scales were smaller, thinner and had a more pronounced triangular shape than those of the populations with winter phenology. SP scales are also significantly darker than those of the two winter populations, which may contribute to raise egg temperature. Being thinner, the scales tend to curl inwards and the eggs are not so well protected from parasitoids, which on the other hand is much lower in this population.

Three egg parasitoid species were found in both Leiria populations. Yet, in contrast with the WP, SP showed a very high proportion of parasitism by generalist species, mostly *Trichogramma* sp., followed by *Oencyrtus pityocampae*, rather than by the specific parasitoid *Baryscapus servadeii*. Specific parasitoids must closely adapt their life cycles and phenology to those of its host (Godfray et al., 1994; Hawkins et al., 1997; Van Nouhuys & Lei, 2004). Regarding the SP, generalist parasitoids were apparently more efficient than the specific *B.*

servadei in exploiting an abundant and fairly stable egg resource. Our results further evidence that the generalist parasitoids showed synchronized emergences with the male flight period of *T. pityocampa*, a fact which may be explained by the exploitation of alternative hosts. By contrast, the specialist parasitoid *B. servadeii* was well synchronized with the flight period of the winter population, but not with the reproductive period of the SP.

In general, *T. pitycampa* egg parasitoids prefer smaller clutches with larger eggs (Pérez-Contreras & Soler, 2004), suggesting that parasitism may also affect the trade-off between the number of eggs and egg size. Yet, at present egg parasitism does not seem to pose a crucial selective pressure, since according to our results, a low rate of parasitism was observed in both Leiria populations, SP and WP.

Results suggest a rapid adaptation of these traits (egg size/ fecundity/ egg covering) in the recently evolved SP. Yet, one should keep in mind that drift alone following the founder effect could be responsible for the differentiation of some phenotypic characteristics. Rapid morphological differentiation and adaptation to local environments has been documented for species invading new habitats (Lambrinos, 2004). Here we document the occurrence of rapid differentiation of the phenotypic characteristics and life traits, for a population remaining *in situ*, using the same habitat and host plant, while having a shifted reproductive season.

6.3. Phenology of the emergence time

Emergences under laboratory conditions were consistent with those observed in the field, SP adults ceasing to emerge about one month before WP adults. Laboratory emergences occur consistently earlier than in the field, probably due to higher and more stable temperatures inside the laboratory.

Through the manipulation of temperature, we accomplished the synchronization of adult emergence of SP and WP, cross-mating reproduction was achieved and hybrid egg masses were obtained. The emergence of the adult hybrids reveals intermediate behaviour when compared to the parental populations. The flight phenology of the hybrids does not match any of the parental populations, and there are no signs of dominance of the parental phenology, thus allowing to conclude that this is a co-dominant trait. Flight patterns were consistent throughout the years of the experiments.

The facts presented strengthen the hypothesis of the occurrence of a saltational shift, leading to a very distinct flight phenology, and hence reproductive period, probably caused by a random mutation in one, or several genes linked to either the emergence phenology, or the termination of diapause.

6.4. Hybrids genetics

The strength of the set of markers used proved to pertinently assign SP and WP individuals to their respective phenological population, using the tested methods. Also hybrids could be easily identified by genetic markers. This result is particularly relevant as it will allow us to keep tracking the population, while searching for possible hybridization that can be expected to increase in the future, with the population geographical expansion. Yet, with both methods, one experimental hybrid is surprisingly assigned to the parental phenological WP cluster. It cannot be exclude that its presupposed genetically summer mother belonged indeed to the WP for its genetic neutral background studied here, but exhibited an aberrant summer phenology. However, a misidentification of this individual, from the experimental crosses to the genotyping seems highly plausible, as such combination of WP genotype - SP phenology was not found in natural populations.

Another individual not completely assigned as F1 hybrid with NewHybrids may be more informative. We demonstrated here that F1 hybrids exhibited in the laboratory an intermediate phenology. If such crosses could happen in nature, then rare back-crosses with the parental winter population could result in a relatively early winter phenology. Indeed, to succeed laboratory crosses, even if the emergence of the summer parents were artificially delayed, we certainly selected parents exhibiting the latest date of emergence among the summer population and the earliest date among the winter population. We therefore maximized the probability to use parents having a hybrid in their own pedigree, as far as such hybrids exist in natural populations and intermediate phenology is quantitatively heritable through generations. But this result could also illustrate the limit of the detection power of the markers we used.

Analysis of larger sampling will be necessary to look for hybrids in natural populations. Simulated genotypes could also be used to precise the level of discrimination possible, using the two methods proposed, between closed classes of genotypes, including backcrosses.

Final remarks

Results here presented clearly show the occurrence of a sympatric allochronic differentiation process that gave rise to the ongoing ecological specialization. The most plausible scenario is that of a phenologically shifted population, recently founded by individuals originating from the local genetic pool, with very limited gene flow presently occurring between both populations. This could be the first stage of allochronic speciation, a process very rarely observed in nature. The SP can be seen as a "phenological race", exposed to different ecological pressures and constraints, that could cause further divergence and still maintain its genetic differentiation. We evidenced the occurrence of a phenological shift that could have led to saltational evolution in this phenological trait, a mechanism very seldom documented, instead of the widely accepted gradual evolutionary model.

Our data further agree with the hypothesis that divergence is probably speeded up and strengthened by exposure to the different ecological pressures now experienced by the SP (Santos et al., 2011b). This work therefore provides a new insight of how ecological divergence may follow the process of allochronic reproductive isolation, a very rarely observed type of sympatric speciation.

Perspectives

Future perspectives for this work should include research on several important aspects of the genetics, ecology and implications of the occurrence and expansion of this population, and the urgent definition of viable management strategies for this recently discovered pest. This population has been observed in high densities ever since its discovery, 15 years ago, with associated damage to the forest and it has expanded especially in the North-South axis. It is thus very important to determine the current geographical distribution and the dispersal patterns of SP as well as its capacity of occurrence in other regions, and to assess the regions at risk using biological, ecological and climatological data to build expansion models. Also, further research about other ecological factors, namely biological control organisms, (predators and parasitoids) that might constitute a constraint for the expansion and build-up of densities of this population, are necessary to study the hypothesis of using biological means to reduce the impact of SP.

Moreover, finding ways to slow down or avoid the expansion, implementing management stragegies in the field might become highly recommendable in the future, should the expansion scenario under climate change reveal to be favourable to the SP. To improve our knowledge of the genetics of the SP, further research on the genetic structure of the sympatric SP and WP, as well as hybridization and introgression phenomena to understand the differentiation of both populations and forecast future evolutionary scenarios. Finally, genomic and transcriptomic resources are currently under development to better understand the genetic bases of the remarkable phenological shift.

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